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Candida glabrata **biofilms: mechanisms of antifungal resistance and matrix role**

Célia Fortuna Rodrigues

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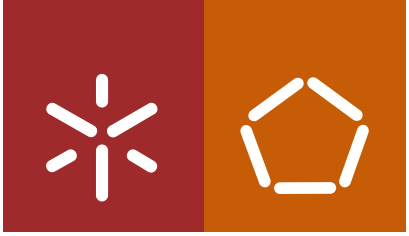

Universidade do Minho
Escola de Engenharia

Célia Fortuna Rodrigues

Candida glabrata biofilms: mechanisms of antifungal resistance and matrix role

Doctoral dissertation for PhD degree in Chemical and Biological Engineering

Thesis supervised by
Professor Mariana Contente Rangel Henriques
Professor Manuel Vilanova

June 2018

STATEMENT OF INTEGRITY

I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagiarism or any form of falsification of results in the process of the thesis elaboration. I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

University of Minho, 14TH JUNE 2018

Full name: CÉLIA FORTUNO RODRIGUES

Signature: _____

Para o Bobo e a Mimi.

Pelo amor imenso que me deram.

"Domino, Quo Vadis?"

41.841108º N 12.532913º E

SENATUS POPULUQUE ROMANUS

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"Somewhere, something incredible is waiting to be known." – Carl Sagan

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"THERE'S no sense in going further—it's the edge of cultivation,
So, they said, and I believed it—broke my land and sowed my crop—
Built my barns and strung my fences in the little border station
Tucked away below the foothills where the trails run out and stop.

Till a voice, as bad as Conscience, rang interminable changes
On one everlasting Whisper day and night repeated—so:
Something hidden. Go and find it. Go and look behind the Ranges—
"Something lost behind the Ranges. Lost and waiting for you. Go!"

Rudyard Kipling (1865–1936), *The Explorer* (1898)

ABSTRACT

Candidiasis are the most common fungal diseases, especially among immunosuppressed, hospitalized and patients with prolonged contact to medical devices. Generally, *Candida albicans* is the most encountered *Candida* spp., however, in the last decades, non-*Candida albicans Candida* (NCAC) spp. have been increasing their responsibility in the cases of candidiasis. In this group, one of the most important species is *Candida glabrata*. This species presents azole resistance, like *Candida krusei* and a quick capacity to develop resistance to echinocandins, similar to *Candida parapsilosis*. Although not having capacity to produce certain important enzymes (e.g. hydrolases), *C. glabrata* has an essential feature that explains part of its recalcitrance to antifungals: the capacity to form biofilms.

The present thesis has the general purpose of increasing the current knowledge on the different and less explored biofilms of *C. glabrata*, their mechanisms of resistance to antifungal drugs, and ultimately contributing to the path into a more consistent optimization and patient response to the present therapies.

Thus, to begin, a method to study the fluconazole's diffusion through the biofilm matrices was developed and then the effectiveness of voriconazole in *C. glabrata* biofilms was determined. It was concluded that voriconazole had higher activity than fluconazole and its diffusion was also deeper. Some changes were noticed in the *ERG3*, *ERG6* and *ERG11* genes expression. Additionally, an association of ascorbic acid (immunostimulator and β-glucan degradator) and fluconazole was applied to biofilms of *C. glabrata*. Biofilms *C. glabrata* were weakened, but not entirely degraded, and the number of cells was higher, due to the assimilation of the glucose derived from the β-glucan hydrolysis.

In the context of polyenes, it was assessed the capacity of *C. glabrata* to form biofilms in the presence of clinical concentrations of amphotericin B. This capacity was confirmed, demonstrating the resistance of this species, even when using drugs with a good antifungal activity. In another approach, the comparison between two amphotericin B formulations – deoxycholate and liposomal – was performed in *C. glabrata* biofilms, and other *Candida* spp.. Both formulations have a good biomass reduction capacity, but the liposomal has a better activity against biofilm cells. The concomitant use of a polyene and an azole was showed to be mostly antagonist, since when applying amphotericin B and posaconazole, the effect in the number of cells, biomass reduction and Fractional inhibitory concentration index (FICI) calculation was not better than using each drug alone.

Caspofungin and micafungin showed to induce great changes in *C. glabrata* biofilm matrices. These echinocandins were evaluated in terms of planktonic and biofilm cell susceptibility, showing to be better in the first case than in the latter. The biomass reduction was very good in both cases, but the variations regarding the quantity of proteins were variable. The amount of β-glucans generally decreased after the echinocandins' contact, in opposition with the other drug's response.

Regarding transcriptomics, firstly a set of genes responsible for the production of matrix components was selected and their expression was assessed, on *C. glabrata* biofilm cells. In a general manner, all genes were overexpressed, but the ones related to the production of β-1,3-glucans revealed a higher expression. It was concluded that the cells invest more in the replacement of these biopolymers than the others and that they might have a greater impact in the biofilm cells lower susceptibility to antifungal drugs. Next, a *C. glabrata* mutant knocked-out on the *MNN2* gene was studied in the biofilm form. The biofilm cells were evaluated in terms of drug susceptibility, cell wall, and the biofilm composition was also determined. There were no visible differences among the cell walls of the strains, but the mutant showed to have a great biomass reduction, after a drug stress. The quantity of mannans of the biofilm cells in the mutant decreased. The biofilm cell walls composition showed an increase of the quantity of β-1,3-glucans in the *C. glabrata* Δ*mnn2*, when compared to the reference strain, possibly compensating the reduction of mannans. This increase in β-1,3-glucans could explain the reduction in the biofilm susceptibility.

The *in vivo* approach permitted an exploration on the drug and immune response of an infection related solely to biofilm cells of *C. glabrata* and then treated with two echinocandins. The results showed that, generally, the immune response in *C. glabrata* is very distinctive from *C. albicans*. The fungal burden was higher in the liver than in the kidneys. Neither caspofungin nor micafungin was effective in fully eradicating *C. glabrata* biofilm cells and it was noticed a predominant mononuclear immune response in the spleen, liver and kidneys of the infected mice.

In summary, the present thesis compiles important data that increase the knowledge on the mechanisms of antifungal resistance of *C. glabrata* biofilms, contributing to the path into a more sustained and defined understanding on how the biofilm matrices and the biofilm cells contribute to more severe infections. Moreover, it is underlined the importance on how these biofilm-derived-infections need to be addressed differently from the the planktonic ones, replacing the current protocols.

Keywords: *Candida glabrata*, biofilm, matrix, antifungal, resistance.

SUMÁRIO

A candidíase é a patologia fúngica mais comum, especialmente em indivíduos imunossuprimidos, hospitalizados e em doentes que necessitam de ser sujeitos a dispositivos médicos durante longos períodos de tempo. Geralmente a *Candida albicans* é a espécie mais identificada, no entanto, nas últimas décadas, a prevalência de espécies de *Candida* não *albicans* sofreu um aumento significativo. Neste grupo, uma das espécies mais importantes é a *Candida glabrata*. Esta espécie possui uma resistência inata aos azóis e uma rápida habilidade de desenvolver resistência às equinocandinas. Embora a *C. glabrata* não tenha capacidade para produzir certas enzimas consideradas fatores de virulência importantes (por exemplo, hidrolases), ela possui uma característica essencial que explica parte da sua resistência aos fármacos antifúngicos: a capacidade de formar biofilmes. A presente dissertação tem como objetivo geral ampliar o conhecimento atual sobre os diferentes, e menos explorados, biofilmes de *C. glabrata*, os seus mecanismos de resistência a fármacos antifúngicos e, por fim, contribuir para uma otimização da resposta do doente relativamente às atuais terapêuticas.

Assim, começou-se por desenvolver um método que permitiu estudar a capacidade de difusão do fluconazol em matrizes de biofilmes de *C. glabrata* e, seguidamente, determinou-se a eficácia do voriconazol nestes biofilmes. Concluiu-se que a atividade deste fármaco era maior que a do fluconazol e sua difusão também era maior. Algumas mudanças na expressão dos genes *ERG3*, *ERG6* e *ERG11* também foram observadas. De seguida, uma associação de ácido ascórbico (imunoestimulador e degradador de β-glucanos) e fluconazol foi aplicada em biofilmes de *C. glabrata,* que foram enfraquecidos, mas não totalmente degradados. Verificou-se um aumento do número de células talvez devido à assimilação da glicose derivada da hidrólise do β-glucano.

Foi também avaliada a capacidade de *C. glabrata* em formar biofilmes em presença de concentrações clínicas de anfotericina B (anf B). Esta capacidade foi confirmada, demonstrando a resistência desta espécie, mesmo usando fármacos com boa atividade antifúngica. A comparação entre duas formulações de anf B - desoxicolato e lipossomal - em biofilmes de *C. glabrata* mostrou que ambas as formulações têm uma boa capacidade de redução de biomassa, mas a lipossómica exibiu uma melhor atividade contra células de biofilme. O uso concomitante de um polieno e de um azole revelou-se sobretudo antagonista, pois ao aplicar anf. B e posaconazole, o efeito na contagem de células, na redução da biomassa e no cálculo do índice fracionário de concentração inibitória não foi melhor do que usar cada fármaco isoladamente.

A caspofungina e a micafungina induziram grandes mudanças na composição bioquímica nas matrizes de biofilme de *C. glabrata*. Estas equinocandinas foram avaliadas em termos de susceptibilidade a células planctónicas e de biofilmes, mostrando-se melhor no primeiro caso do que no segundo. A redução da biomassa foi muito boa em ambos os casos, mas as variações quanto à quantidade de proteínas foram inconstantes. A quantidade de β-glucanos geralmente diminuiu após o contato das equinocandinas, em oposição à resposta de outros fármacos de classes diferentes.

Foi, depois, selecionado um conjunto de genes responsáveis pela produção de componentes da matriz e foi avaliada a sua expressão, nas células de biofilme de *C. glabrata*. De forma geral, todos os genes foram sobreexpressos, mas os relacionados com a produção de β-1,3-glucanos tiveram uma expressão mais elevada. Concluiu-se que as células investem mais na substituição desses biopolímeros do que na dos demais, podendo ter um maior impacto nas células de biofilme, induzindo uma menor susceptibilidade a fármacos antifúngicos. Para completar este estudo avaliou-se a capacidade de formação de biofilme de um mutante de *C. glabrata* sem o gene *MNN2*. Não se detetaram diferenças visíveis entre as paredes celulares das estirpes, mas o mutante apresentou uma grande redução de biomassa, após o *stress* provocado pelos fármacos. A quantidade de mananos das células do biofilme no mutante diminuiu e a composição das paredes celulares do biofilme mostrou um aumento da quantidade de β-1,3-glucanos na *C. glabrata* Δ*mnn2*, possivelmente compensando a redução de mananos. Este aumento nos β-1,3-glucanos poderá explicar a redução da susceptibilidade do biofilme aos fármacos.

O ensaio *in vivo* permitiu estudar a resposta imune e das equinocandinas, relativamente a uma infeção de *C. glabrata* originada exclusivamente com células de biofilme. Os resultados mostraram que a resposta imune em *C. glabrata* é muito distinta da de *C. albicans*. A carga fúngica foi maior no fígado do que nos rins e nem a caspofungina nem a micafungina foram efetivas na erradicação total das células de biofilme de *C. glabrata*.

Em resumo, a presente dissertação compila dados importantes que acrescentam conhecimento sobre os mecanismos de resistência antifúngica aos biofilmes de *C. glabrata*, contribuindo para uma compreensão mais sustentada e definida sobre como as células e as matrizes de biofilmes contribuem para infeções mais graves de *C. glabrata*. Além disso, sublinha a importância das infeções derivadas do biofilme, que deveriam ser abordadas de maneira diferente das planctónicas, sendo importante ajustar os protocolos atuais de terapêutica.

Palavras-chave: *Candida glabrata*, biofilme, matriz, antifúngico, resistência.

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

ABBREVIATIONS AND ACRONYMS

AA	Ascorbic Acid
Abs	Absorbance
ACT	Actin gene
ABC	ATP binding cassette transporter gene superfamily
ACE	Putative transcription factor
ADH	Alcohol dehydrogenase gene
Als	Agglutinin like sequence protein
AIDS	Acquired Immune Deficiency Syndrome
ALS	Agglutinin like sequence gene
AmB	Amphotericin B
AmB-Deox	Amphotericin B deoxycholate
AmB-L	Liposomal Amphotericin B
ANOVA	Analysis of variance
APC	Allophycocyanin
anti-CD11	anti-mouse glycoprotein CD11
anti-CD16/36	anti-mouse low affinity IgG Fc receptor III (FcR III)
anti-CD86	anti-mouse immunoglobulin CD86
anti-CR3-RP Ab	Polyclonal anti-CR3-RP antibody
anti-F4/80	anti-mouse glycoprotein F4/80
anti-GR1	anti-mouse granulocyte-differentiation antigen-1
AQR	Proton antiporter gene
Aqr	Proton antiporter protein
ARO	Tyrosine gene
ATCC	American Type Culture Collection
ATG	Predicted role in non-selective autophagy gene
Awp	Adhesin-like protein
BCA	Bicinchoninic Acid
Bcr	Transcription factor
BDL	Below the detection limit
BGL	1,3-β -glucanosyltransferase gene
Bgl	1,3-β -glucanosyltransferase protein
BSA	Bovine Serum Albumin
BV	Brilliant violet
CD	Cluster of differentiation
CDC	Centers for Disease Control and Prevention
CDR	ATP binding cassette transporter gene

CEMUP	Centre of Materials of the University of Porto
CFU	Colony forming units
CHROMagar	Chromogenic media agar
CHS	Chitin synthase-encoding gene
CLSM	Confocal Laser Scanning Microscopy
CR3-RP	Complement receptor 3-related protein
Csf	Caspofungin
CSH	Surface hydrophobicity gene
Cst	Transcription factor, bZIP domain-containing protein
CTA	Catalase gene
Cta	Catalase protein
CUG clade	Leucine tRNA; CTG codon-amino acid adaptor activity (nonstandard translation for CUG; triplet codon-amino acid adaptor activity that recognizes a CUG codon)
CV	Crystal Violet
Cy	Cychrome 7
DC	Denditric cell
DHA	Putative polyamine transporter; MFS-MDR family (drug H^+ antiporter)
cDNA	Complementary Deoxyribonucleic Acid
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic Acid
DMSO	Dimethyl sulfoxide
dNTP	Desoxynucleoside triphospahte
DSPG	Distearylphosphatidyl glycerol
ECM	Extracellular matrix
ECOFF	Epidemiological cut-off value
EPA	Epithelial adhesin gene
Epa	Epithelial adhesin protein
EUCAST	European Committee on Antimicrobial Susceptibility Testing
e.g.	(*Exampli gratia*) for example
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
et al	(*Et alii*) and others
ERG	Ergosterol biosynthesis gene
Erg	Ergosterol biosynthesis protein
Evo	Evolved strain
FAA	Fatty acid activator gene
FC	Fold change
FCT	Fundação para a Ciência e Tecnologia
FcγR	Receptors for the Fc region of immunoglobulin G

FDA	Food and Drug Administration
FEDER	Fundo Europeu de Desenvolvimento Regional - Portugal 2020
FICI	Fractional inhibitory concentration index
FITC	Fluorescein isothiocyanate
FKS	Glucan synthase like gene
Fks	Glucan synthase like protein
FLO	Flocculation gene
Flu	Fluconazole
FLR	Multidrug transporter of the major facilitator superfamily (involved in 5-flucytosine resistance)
FVD	Fixable Viability Dye
FTIR-ATR	Fourier-transform infrared spectroscopy – attenuated total reflection
Ftr	Putative ferrous iron transmembrane transporter involved in iron uptake
FUR	Putative uracil phosphoribosyltransferase gene
g	Centrifugal force relative to the gravitational force of the earth
GAFFI	Global Action Fund for Fungal Infections
GAS	1,3-β -glucanosyltransferase gene
GCAL	g-carbonic anhydrase gene
GI	Gastrointestinal
GOF	Gain-of-function mutations
GPI	Glycophosphatidylinositol anchor protein
Gpx	Glutathione peroxidase protein
HBSS	Hanks' Balanced Salt Solution
HE	Hematoxylin-eosin
HIV	Human Immunodeficiency Virus
HLP	Haemolytic like protein gene
HPLC	High-performance liquid chromatography
HSP	Heat shock family protein gene
Hsp	Heat shock protein
i.e.	(*id est*) that is
Ig	Immunoglobulin
i.p.	intraperitoneal
Il	Interleukin
ITS	1-phosphatidylinositol-4-phosphate 5-kinase gene
i.v.	intravenous
KHN	1,6-β -D-glucan biosynthetic gene
KO	Knock-out KO
kre	killer resistant mutations
KRE	1,6-β -D-glucan biosynthetic gene
LC	Liquid chromatography

LDH	Lactate Dehydrogenase
LIFE	Leading International Fungal Education
LIP	Lipase gene
Log	Logarithm
M	Molar or molarity
MALDI-TOF MS	Matrix assisted laser desorption ionization-time of flight mass spectrometry
MAR	Multi antimicrobial resistance
MBEC	Minimum biofilm eradicatory concentration
Mcf	Micafungin
MDR	Multidrug resistance gene
MET	Multidrug endosomal transporter
MFC	Minimum fungicidal concentration
MFI	Mean fluorescence intensities
MFS	Major facilitator family
MIC	Minimum inhibitory concentration
min	Minute
MCK	Mitogen activated kinase gene
MHC	Major histocompatibility complex (class I or II)
MNN	α -mannosyltransferase gene
Mpk	MAP kinase phosphatase signaling protein
MS	Mass spectrometry
MSH	Putative DNA mismatch repair factor
MSK	Muscle creatine kinase gene
Msn	Putative transcription factor similar to *Saccharomyces cerevisiae*
mRNA	Messenger Ribonucleic Acid
NCAC	non-*Candida albicans Candida*
ND	Non-Detected
NIH	National Institutes of Health
NMR	Nuclear magnetic resonance
NS	Non-significant
NRT	Non-transcriptase reverse controls
OD	Optical density
PAS	Periodic-acid Schiff
PBS	Phosphate buffered saline
Pcz	Posaconazole
PCR	Polymerase Chain Reaction
PDR	Pleiotropic drug resistance gene
PE	Phycoerythrin
Pep	Vacuolar aspartyl proteinase
PerCp Cy5.5	Peridinin Chlorophyll protein
Phr	Pleiotropic drug resistance protein

pH	Potential hydrogen
PHR	ATP binding cassette transporter gene
PI3K	Phosphoinositide 3-kinase
PI3P	Phosphatidylinositol 3-phosphate
PLs	Phospholipases
PMNs	Polymorphonuclear leucocytes
PMS	Phenazine methosulfate
PMSF	Phenylmethylsulfonyl fluoride
PNA	Peptide Nucleic Acid
PUP	Pdr1 UPregulated
Qdr	Drug H^+ antiporter of the major facilitator superfamily protein
r	Pearson Correlation Coefficient
Ras	GTPase activity protein
Rif	Subtelomeric silencing protein
RHOE	Reconstituted Human Oral Epithelium
RIF	Sub-telomeric silencing gene
RNA	Ribonucleic Acid
RND	Resistance Nodulation Division
rpm	Rotation per minute
RPMI-1640	Roswell Park Memorial Institute Medium
rRNA	Ribosomal Ribonucleic Acid
ROS	Reactive oxygen species
RT- qPCR	Real-time Polymerase Chain Reaction
RVOE	Reconstituted Human Vaginal Epithelium
S.A.	Anonymous society
SAP	Secreted aspartly proteinase gene
Sap	Secreted aspartly proteinase protein
SDA	Sabouraud dextrose agar
SDB	Sabouraud dextrose broth
SD	Standard deviation
SD-his	Sabouraud dextrose agar without histidine
SEM	Scanning Electron Microscopy
SENTRY	Antimicrobial Surveillance Program
SKN	Predicted transcription factor gene
Skn	Predicted transcription factor protein
SIR	Putative NAD^+ dependent histone deacetylase of the sirtuin gene family and involved in subtelomeric silencing
Sir	Subtelomeric silencing protein
SMR	Small Multidrug Resistance
SNP	Single nucleotide polymorphism
SNQ	ATP binding cassette transporter gene
Snq	ATP binding cassette transporter protein

SOD	Superoxide dismutase gene
SOD	Superoxide dismutase protein
STB	Predicted sequence-specific DNA binding transcription factor
SVF	Stromal vascular fraction
Swi/Snf complex	ATPase product of the yeast *SNF2,* component of the chromatin remodelling
TNF-α	Tumor necrosis factor alpha
TORC	Rapamycin and nutrient-sensitive TOR complex
TPO	Predicted polyamine transporter of the major facilitator gene superfamily (drug H^+ antiporter)
Tpo	Predicted polyamine transporter of the major facilitator s uperfamily (drug H^+ antiporter) protein
TSA	Predicted thioredoxin peroxidase gene
Tsa	Thioredoxin peroxidases
TRR	Thioredoxin gene
Trr	Thioredoxin reductases
TRX	Thioredoxin gene
Trx	Thioredoxin protein
UPG	1,6-β -D-glucan biosynthetic gene
USA	United States of America
U	Units
V	Voltage
Vcz	Voriconazole
Vps	Putative phosphoinositide 3-kinase involved in iron homeostasis
v/v	Volume to volume
w	Weight
XOG	Glucan endo-1,6 and 1,3-β -glucosidase gene
Xog	Glucan endo-1,6 and 1,3-β -glucosidase protein
XTT	2,3bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]2-Htetrazolium hydroxide
YAK	Putative serine-threonine protein kinase gene
Yak	Putative serine-threonine protein kinase
YAP	bZIP domain-containing gene
Yap	bZIP domain-containing protein
YNB	Yeast Nitrogen Base
Yor	Putative ATP binding cassette transporter protein
YPS	Yapsin aspartic protease gene family
Yps	Yapsin aspartic protein
ZAP	Zinc regulated gene

SYMBOLS

%	Percent
®	Registered trademark
º	Degrees
ºC	Celsius degrees
p	Significance value
t	Time
™	Trademark
V	Volume

SCOPE, AIM AND OUTLINE OF THESIS

SCOPE OF THE THESIS

Considering the increasing number of immunosuppressive diseases (e.g. cancer, HIV), responsible for longer hospitalization periods and for the need of the use of several medical devices, the appearance of nosocomial infections has also been ascending. Among them, fungal diseases, as candidiasis, have shown to be of great importance, due to an associated higher drug resistance, especially when related to cells from communities of microorganisms, the biofilms. *Candida* spp. biofilms are incredibly more recalcitrant to the antifungal treatment than the planktonic cells that originated then. The exopolymeric compounds that form the matrices of these communities are a strong source of protection from any chemical or, even, physical aggressions.

Globally, the most common species responsible for the cases of candidiasis continues to be *Candida albicans,* however, in the last decades, NCAC spp, as *C. glabrata,* have rapidly emerged. *C. glabrata* is a distant species of the *Candida* genus, closer to *Saccharomyces cerevisiae.* It is recognized to have an innate resistance to the azole class (as *C. krusei* and *C. auris*) and to rapidly develop resistance to echinocandins (as *C. parapsilosis*). *C. glabrata* forms biofilms with a high quantity of polysaccharides, proteins and other constituents, but this profile quickly changes with any environmental stress, resulting in thicker biofilms, more protective to the cells. This aptitude conditions the therapeutic responses, enabling the evolution of local to recurrent or systemic and serious candidiasis.

In spite of all the developments observed in the comprehension of the mechanisms of resistance to antifungals in *C. glabrata*, there is still much work to do to reach the full understanding on this matter. The characterization of the biochemical changes on the biofilms that induce further genetic and mechanistic responses in stress conditions, may impact the future therapeutic protocols and clinical outcomes of the treatment of infections of *C. glabrata* biofilms.

AIM OF THE THESIS

The present thesis has the general purpose of increasing the current knowledge on the mechanisms of resistance of *C. glabrata* biofilms to antifungal agents and to understand the role of the biofilm matrix in this resistance.

It was a purpose to specifically focus on the matrix components to circumscribe the resistance phenomena, in order to treat matured biofilms of *C. glabrata.*

Hence, the first goal was to determine the capacity of azoles to diffuse through the biofilm matrices and to effectively eliminate *C. glabrata* biofilms. Then, the effectiveness of voriconazole to be used as a treatment to oral candidiasis was assessed. This second-generation azole was compared to the first (fluconazole) in terms of capacity of cells' reduction and biomass decrease and diffusion in the matrix. Three selected *ERG* genes expression were also studied. Also, the association of fluconazole and ascorbic acid was estimated to be possibly applied in a clinical line to treat oral mucositis.

Secondly, the work was focused on determining the amphotericin B (AmB) drug response and matrix alterations on *C. glabrata* biofilms. The first work was to determine if *C. glabrata* had the ability to form biofilms in presence of clinical concentrations of AmB and, in a second turn, the drug effective response of two AmB different pharmaceutical formulations were evaluated, with the objective to conclude which one would be more clinically interesting to treat *C. glabrata* biofilms, but also other *Candida* spp.. Finally, an association of AmB and posaconazole (recent triazole) was assessed as a possible alternative drug combination to treat these biofilms.

The third goal was to explore the capacity of echinocandins to eliminate biofilms of *C. glabrata* (and other *Candida* spp.). The biochemical modifications induced in the biofilm matrices by micafungin and caspofungin were evaluated (proteins, β-1,3-glucans) and related to possible mechanisms of resistance.

The fourth objective was to make a transcriptomic approach to *C. glabrata* biofilms. Primarily, a set of genes associated to the production of components of the matrices were carefully selected and their expression was evaluated, through RT-qPCR, with and without the stress induced by fluconazole, amphotericin B, caspofungin and micafungin. In a second approach, a *C. glabrata* mutant knocked-out in the *MNN2* gene, responsible for the production of mannans, was studied in terms of the susceptibility to antifungals and the resistance profile of the biofilms.

The fifth and final goal of this thesis was to study *C. glabrata* biofilm cells in an *in vivo* mouse model. For that, CD1 mice were infected intravenously with cells derived exclusively from *C. glabrata* biofilms and the infection treated in caspofungin or micafungin. The drug and immune response was, then, evaluated and analysed.

OUTLINE OF THE THESIS

The present thesis reports the work performed at the LIBRO - Laboratório de Investigação em Biofilmes Rosário Oliveira (Laboratory of Investigation of Biofilms Rosário Oliveira), Centre of Biological Engineering, University of Minho, Braga and Manuel Vilanova's Immunobiology Laboratory at Abel Salazar Institute of Biomedical Sciences and i3S - Institute for Investigation and Innovation in Health, Portugal, under the supervision of Professor Doctor Mariana Henriques and Professor Doctor Manuel Vilanova.

This thesis is organized into nine chapters:

The **Chapter I** is a general introduction in which the biofilms of *C. glabrata* are discussed and all the issues on biofilm matrix formation and resistance mechanisms are briefly discussed. The biology and epidemiology of this pathogen is presented and related with its important capacity of biofilm forming and, thus, to be recalcitrant to pharmacotherapies. Additionally, new alternatives to the antifungal treatments, are concisely described.

The next chapters (Chapters II to VIII) correspond to different parts of the research work performed, containing the main experimental results.

In the **Chapter II** it is reported the work performed in the analysis of the biofilm matrices of *C. glabrata.* A new HPLC method was developed to detect fluconazole and to quantify it within the matrices of *C. glabrata*. Other methods (FTIR, MALDI-TOF), that have failed to do this approach, are also explained and addressed.

The **Chapter III** describes the work with *C. glabrata* biofilms and azoles.

In the first part it is presented a report studying the effectiveness of voriconazole on biofilms of *C. glabrata*, in comparison to other azole (fluconazole) and how the capacity of the diffusion in the biofilm matrices can be related to this effectiveness.

In the second part, an association of fluconazole and ascorbic acid (a known immunoatimulator and β-glucan degradator) is assessed against these biofilms.

In **Chapter IV** it is presented the effect of polyenes on *C. glabrata* biofilms.

The first part presents the study of the development of *C. glabrata* biofilms during the use of amphotericin B.

The second part is a comparison between two pharmaceutical formulations of amphotericin B - liposomal and deoxycholate –, in order to evaluate which could be clinically more appealing against biofilms of *C. glabrata*.

The third part is an evaluation of the association of an azole to a polyene: posaconazole and amphotericin B, and its possible application in therapy.

The **Chapter V** is an approach to echinocandins and its effect on biofilms of *C. glabrata* in terms of effectiveness of treatment and biochemical alterations in the matrices.

The **Chapter VI** is a transcriptomic approach.

In the first part, eight genes - related to the production of matrix components - are studied trough RT-qPCR after a drug stress induced in biofilm cells of *C. glabrata*, caused by fluconazole, amphotericin B, caspofungin and micafungin.

The second part is an evaluation on how the knock out of the *MNN2* gene (responsible for the mannans production) would modulate the antifungal resistance of the biofilms of *C. glabrata*.

Chapter VII includes an *in vivo* approach of biofilms infections of *C. glabrata.* CD1 mice were intravenously infected with biofilm cells of *C. glabrata* and treated with caspofungin and micadungin. The results were evaluated in terms of drug and immune responses.

In **Chapters VIII** and **XIX** are presented the general discussion and conclusions, and the work perspective, respectively.

Chapter I
State of the Art

The work presented in this chapter was adapted from:

Rodrigues, C.F.; Silva, S.; Henriques, M. *Candida glabrata*: a review on its features and resistance. *European Journal of Clinical Microbiology Infectious Diseases*. **2014**, *33*(5):673-88. doi: 10.1007/s10096-013-2009-3

Rodrigues, C.F.; Rodrigues, M.E.; Silva, S.; Henriques, M. *Candida glabrata* Biofilms: How Far Have We Come? *Journal of Fungi*. **2017**, *3*, 11. doi:10.3390/jof3010011

I
State of the Art

I.1 Introduction

I.1.1 The Biofilm Era

From 1880 until the middle of the twentieth century, conventional microbiology was in the so-called "pure culture period" [1]. Microorganisms were viewed merely as free-floating single cells (i.e. planktonic) and most studies focused only the proliferation of bacteria in liquid media on agar plates or in test tubes. In fact, today this reality seems very peculiar, since it is estimated that less than 0.1% of the total microbial biomass has planktonic phenotypes [2,3].

The first documented scientific report about a biofilm was written in 1683 by Antonie van Leeuwenhoek in the Royal Society of London [4,5]. van Leeuwenhoek described its first observation of surface-associated aggregated bacteria, designating them as the "animals" present in the plaque on teeth [6]. Later, in 1933, Henrici took photomicrographs of aggregating bacteria and finally perceived that "the most part water bacteria are not free floating organisms, but grow upon submerged surfaces" [7]. Thirty years later, the term biofilm was published by Rogovska et al in Microbiology-USSR [8], in an environmental microbiology publication [9]. In 1977, Høiby described aggregates (heaps) of *Pseudomonas aeruginosa* in the lungs of chronically infected cystic fibrosis patients [10].

Since then, the reports in the medical microbiology field began to acknowledge biofilms. Costerton and colleagues portrayed a medical biofilm as the surface adhering bacteria embedded in a "glycocalyx" (matrix) (1978), and, in 1981, they used the term "biofilm" for the first time, to describe it [11]. The phenomenon was revised and re-defined as a matrix enclosed mode of growth, in 1987, by the same authors [12]. The biofilm form of growth phenotype was finally accepted and considered significant to microbiology in 1993, by the American Society for Microbiology [13]. Six years later, Costerton et al [14] gave the final characterisation of biofilm: "a structured community of bacterial cells enclosed in a self-produced polymeric matrix, adherent to a surface" (Figure I.1).

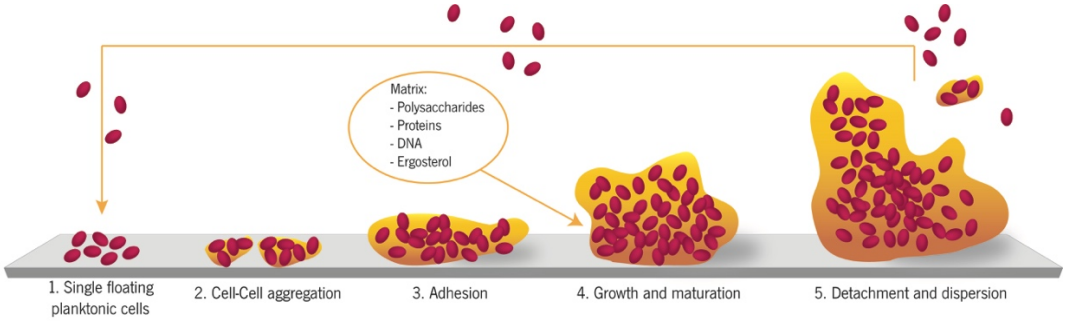


Figure I.1. Scheme of the biofilm formation.

I.1.2 The biofilm form and the relation to chronic and severe infections

Biofilms are believed to be contemporaneous of the first bacteria and fungi and they are classically responsible for chronic persistent infections. Prior to the antibiotic period, simple bacterial or fungal infections had high health risks [15]. People rapidly died of pneumonia and other acute infections that are now more easily cured using antibiotics. The development of antibiotics led a higher rate of slow-progressing infections, arising in all age groups where patients experienced fever, distress, and other typical clinical signs of infection. In these cases, microorganisms could not be detected, the treatments were ineffective, resulting in persistent infections [15]. The identification of the foundations of these very particular infections was a series of *in vitro* and *in vivo* observations made in the 1980s [16], which indicated that the aggregation of bacteria was the cause of slow-progressing infections [17]. Yet, biofilms and their high tolerance to drugs had been discovered 300 years earlier, by Van Leeuwenhoek [6]. In 1684, this researcher documented that the bacteria within the plaque of teeth were more resistant to vinegar than bacteria found outside the plaque, which were killed [15]. Nowadays, it is well known that biofilms have an extreme tolerance to antimicrobial agents. The biofilm antibiotic tolerance must not be confused with antibiotic resistance. Though any microorganism within a biofilm tend to survive the antibiotic treatment, generally, it becomes susceptible to the treatment when the biofilm is disrupted [18]. Chronic infections have a much slower evolution than acute infections. The symptoms associate to them are frequently unclear [16] and they are very challenging, or even impossible to treat with antibiotics. The associated chronic inflammation is usually characterized by an adaptive inflammatory response, controlled by mononuclear leucocytes, IgG antibodies and, frequently, continuous recruitment of polymorphonuclear leucocytes (PMNs). Chronic infections are very frequent in immunosuppressed patients or conditions that cause deficiencies in the primary defensive barriers (innate immunity) [15]. The same happens with opportunistic infections (e.g.

nosocomial), that commonly turn into very serious systemic infection, that put in danger the life of the patients.

I.1.3 Biology of Candida glabrata

Historically, *Candida glabrata* strains were originally classified in the *Torulopsis* genus due to its lack of filaments forms formation. However, in 1978, it was determined that the ability to form hyphae and/or pseudohyphae was not a reliable distinguishing factor or members of genus *Candida* spp. and it was proposed that *Torulopsis glabrata* should be classified in the genus *Candida* spp.,due to its human pathogenicity [19]. In fact, in contrast to other *Candida* spp., *C. glabrata* is not polymorphic and, excepting stressful conditions which induce pseudohyphae forming [20–22], this species grow only as blastoconidia. In 2004, *C. glabrata* (CBS138/ATCC2001, American Type Culture Collection) genome was sequenced [23] and showed that this species has a common ancestor with *Saccharomyces cerevisiae* [24,25]. Though, *C. glabrata* has lost more genes, decreasing traces of duplication to a minimum and leading to the complete loss of some metabolic pathways [23]. *C. glabrata* has a critical distinguishing characteristic – a haploid genome -, in opposition to the diploid genome of *Candida albicans* and other *Candida* spp. [26] and several deficiencies in the mating pathway have been also identified [27]. Also, *C. glabrata* are noticeably smaller (1-4 μm) than *C. albicans* (4-6 μm), *Candida tropicalis* (4-8 μm), and other *Candida* spp. blastoconidia [28] (Figure I.2 and Table I.1).

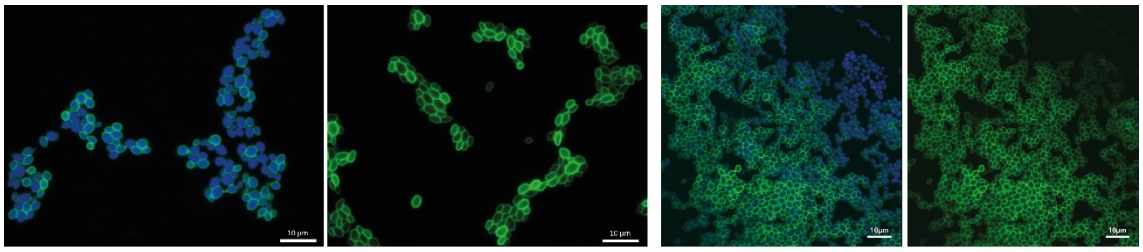


Figure I.2. *Candida glabrata* cells in planktonic and biofilm forms.

In Sabouraud Dextrose Agar (SDA) culture medium, *C. glabrata* strains forms glistening, smooth, and cream-colored colonies, which are relatively indistinguishable from those of other *Candida* spp. except for their relative size, which can be quite small [29]. On CHROMagar™ *Candida* (CHROMagar™, Paris, France), a differential agar medium, it is possible to distinguish a number of different *Candida* spp. by colour, as a result of distinct biochemical reactions, *C. glabrata* colonies

appear white, pink to purple (Table I.1), in contrast to *C. albicans* colonies which are blue-green. Concerning the biochemical reactions of *Candida* spp., *C. glabrata* ferments and assimilates only glucose and trehalose, contrary to *C. albicans*, which ferments and/or assimilates a high number of sugars, excluding sucrose [30,31] (Table I.1).

Table I.1. Characterization of *Candida glabrata* general features [19,23]

Feature	***Candida glabrata* characteristics**
Ploidy	Haploid
Hyphae/Pseudohyphae	Absent/in certain conditions
Colonies on SDA	Very small/cream-color
Cells' size	1-4 μm
Growth on CHROMagar™	White, pink/purple
Biochemical reactions	Ferments and assimilates glucose and trehalose
Virulence	Opportunistic pathogen
Major sites of infection	Vaginal, Oral, Disseminated
Biofilm formation	Yes
Major adhesins	Lectins
Mating genes	Present
Sexual cycle	Unknown
Clonal population structure	Yes
Phenotipic switching	Present
Auxotrophy	Niacin, Thiamin, Pyridoxine
Mitochondrial function	Petite positive
Epidemiology of infection	Principally nosocomial (except vaginal) Frequently mixed fungal infection Immunocompromised or debilitated host Specific risk factors: prolonged hospitalization, prior antibiotic use, use of fluconazole, patient exposure, hand carriage by hospital personnel

Natural auxotrophy of *C. glabrata*, such as the disability to synthesize nicotinic acid, pyridoxine and thiamine [34] and the inability to use galactose or engineered aminoacid auxotrophy [32,33,35], is generally compensated by the mammalian host environment. It is known that the improvement of genes involved in cell wall organization occurred in *C. glabrata*, possibly facilitating adherence to a broad spectrum of surfaces. The gain or loss of these genes may be crucial for a functional differentiation between species and could be related to *C. glabrata* adaptation as a mammalian commensal [27,32,35,36].

Regarding the iron acquisition from host sources - acknowledged as a crucial virulence determinant for *C. glabrata* -, less is known. Very recently, Gerwien ald collegues [37] have determined that this

species is much more restricted than *C. albicans*, missing, for instance, the capacity to grow on transferrin and hemin/hemoglobin. In its place, *C. glabrata* uses ferritin and non-protein-bound iron ($FeCl_3$) as iron sources in a pH-dependent manner. As in other fungal pathogens, iron-dependent growth demands the reductive high affinity (HA)-iron uptake system. This highly conserved uptake mechanism generally relies on initial ferric reduction by cell-surface ferric reductases. The *C. glabrata* genome holds only three such putative ferric reductases, which were found to be unnecessary for iron-dependent growth. Moreover, unlike to *C. albicans* and *S. cerevisiae*, the authors also noticed no surface ferric reductase activity in *C. glabrata*. As an alternative, extracellular ferric reduction was found in this and the other two other fungal species, which was largely dependent on an excreted low-molecular weight, non-protein ferric reductant, possibly compensating the loss of surface ferric reductase activity in the HA-iron uptake system [37]. The regulation of the iron homeostasis has been showed by Sharma et al [38]. Previoulsy, it was explained that the phosphoinositide 3-kinase (PI3K), phosphorylates phosphatidylinositol and produces phosphatidylinositol 3-phosphate (PI3P), and it was associated in protein trafficking, intracellular survival and virulence in *C. glabrata*. The authors confirmed that PI3-kinase (Vps34) is crucial for conservation of cellular iron homeostasis. Vps34 showed to be associated to the regulation of iron acquisition, accentuating its function in vesicular trafficking as a central determinant. Upon Vps34 disruption, iron homeostasis genes were differentially expressed. Iron-limitation showed to induce the expressing the iron permease Ftr1 mostly on the cell membrane, and to iron-excess via internalization of the plasma membrane-localized Ftr1 to the vacuole and Vps34 was essential for this process.

Finally, whereas *C. albicans, Candida parapsilosis* and *C. tropicalis* are moderately closely related species of the CUG clade, which share a unique codon exchange from leucine to serine, *C. glabrata* is actually a “misnomer”, for it is really much more closely related to the *S. cerevisiae* than to *C. albicans* [23,27]. As mentioned, oppositely to the other *Candida* spp., but equally to its “cousin” *S. cerevisiae*, *C. glabrata* is strictly haploid and typically grows only in the yeast form [36].

Examples of factors causing stress to *C. glabrata* (intrinsic or habitat sourced) are nutrient limitation, oxidative response, competition with other microorganisms, agents as protective mechanisms, and the impossibility to generate resistant spores [39]. Hence, the somatic cells require holding viability on surfaces outside the host, demanding a high capacity of stress tolerance. The reports show that, comparing to other *Candida* spp., *C. glabrata* appears to be one of the more robust *Candida* spp., being able to survive on inanimate surfaces for more than 5 months, while

the viability of *C. albicans* is limited to 4 months and *C. parapsilosis* cells die after 2 weeks [39, 40].

I.1.4 Epidemiology and virulence factors of Candida glabrata

Despite their clinical implication and considerable human health burden, fungal infections continue to be under-appreciated. The Leading International Fungal Education (LIFE) portal has enabled, since 2013, the assessment of the burden of serious fungal infections country by country for over 5.7 billion people (>80% of the world's population). A fresh review analyzed 43 important reports[39]. A recent global study have found 3,000,000 cases of chronic pulmonary aspergillosis, ≈223,100 cases of cryptococcal meningitis, ≈700,000 cases of invasive candidiasis, ≈500,000 cases of *Pneumocystis jirovecii* pneumonia, ≈250,000 cases of invasive aspergillosis, ≈100,000 cases of disseminated histoplasmosis, over 10,000,000 cases of fungal asthma and ≈1,000,000 cases of fungal keratitis occur annually [39]. The Global Action Fund for Fungal Infections (GAFFI) indicate that an insufficient patient and clinician awareness, low number of trained clinicians, small index of diagnostic suspicion, deficient laboratory identification capacity and diagnostic tools, and few treatment options contribute to an under-recognition of the true burden of fungal diseases [40,41].

In fact, for many years, *C. glabrata* was considered a non-pathogenic saprophyte of the normal flora of healthy individuals and certainly not readily associated with serious infections in humans. Though, following the widespread and increased use of immunosuppressive therapy together with broad–spectrum antibiotic therapies, the frequency of mucosal and systemic infections caused by *C. glabrata* arose significantly [42,43]. Whilst mycological studies have shown that *C. albicans* represents approximately 80% of the clinical isolates, in the last decades, the number of candidiasis and fungemia cases due to non-*Candida albicans Candida* (NCAC) spp. has meaningfully raised, namely in what concerns *C. glabrata* strains [19,34,44–50].The incidence of this species is higher in adults than in children, and is low in neonates [51,52]. In 2006, the results on the European Confederation of Medical Mycology survey indicated that the frequency rates of candidiasis attributed to *C. glabrata* were around 14% [53] and 15% of all *Candida* spp.-related systemic bloodstream infections [54,55]. This outcome was extremely important since that, compared to other *Candida* spp. infections, the mortality rate associated with *C. glabrata* is the highest [56].

Subsequently to the introduction of the highly active antiretroviral therapy, a reduction in the percentage of oropharyngeal infections, the colonisation by *Candida* spp., and a decline in the frequency of fluconazole resistance in patients with HIV infection have been recorded [57].

However, *Candida* spp. are still the most frequent cause of systemic mycosis in our time [58,59]. During 1995–1996 and 1997–1998, a national programme of surveillance of bloodstream infections in the USA [60], and the SENTRY international programme of surveillance of bloodstream infections in the USA, Canada, and South America [61], showed the importance rising of NCAC spp., which accounted for between 44% and 48% of cases of fungemia. Among NCAC spp., *C. glabrata* clearly stood out, with an increase through the study period in all three geographical regions, becoming the second most frequent species after *C. albicans* in the USA and Canada [60,61] and with a mortality rate associated of 49% in the bloodstream infections, in a retrospective series of 139 cases [62]. In the European SENTRY programme, *C. glabrata* was the third NCAC spp. most common, after *C. parapsilosis* [63]. By contrast to the USA's SENTRY programme, NCAC spp. were generally more susceptible to fluconazole. Nonetheless, a continuous scrutiny is required to confirm the pattern, as it is known that this may be not accurate in present days, as most *C. glabrata* and *Candida krusei* strains intrinsically are resistant to this drug [60,61,63–68].

Until recently, few studies have evaluated independent risk factors associated with nosocomial *C. glabrata* acquisition and the subsequent infections. Little is known about the hospital reservoirs of *C. glabrata*, however with *C. albicans*, probable sources include a complex interaction of environmental and human pools [69]. Vasquez and colleagues [70] revealed that patients with a new acquisition of *C. glabrata* had extended and repeated hospitalizations prior to antifungal use, compared to patients with no *Candida* spp. exposition. Likewise, *C. glabrata* has been often isolated from patients with oral candidiasis, alone or coupled with *C. albicans* clinical isolates [30,71] and related to recurrent systemic infections [72,73]. The propensity of *C. glabrata* for dissemination and the high mortality associated can be linked to the virulence factors that this species exhibits, namely the elevated rates of resistance to the most traditional antifungals.

The relatively nonpathogenic nature of *C. glabrata* in animal models [74,75] suggests that it has only few virulence attributes. However, the high mortality rate and the rapidity spread of disease would argue the contrary [19]. In fact, in opposition to inability to form hyphae and/or pseudohyphae and to secret proteases, *C. glabrata* retains many virulence factors such as the capacity to secrete phospholipases, lipases and haemolysins, that contribute for an extreme aggressiveness resulting in a low therapeutic response and serious recurrent candidiasis [44,76]. Still, its most worrying virulence factor is the strong capability to form biofilms [44,77,78].

I.1.4.1 Adhesion capacity

Candida glabrata clinical isolates have the ability to form a compact biofilm structure in different multilayers [77,78], with proteins, carbohydrates (e.g. β-1,3-glucans, β-1,6-glucans) and ergosterol into their matrices [77–79]. The first step on biofilm development is adhesion and/or colonisation of yeast cells to a surface [30,44]. *Candida glabrata* is capable to colonize biotic or abiotic surfaces, where it develops as multilayered biofilm structure [77,80], despite of not being able to form filaments. This adhesion step is extremely important, not only in the biofilm formation but also in the infection process, and the extent of adhesion is dependent on *C. glabrata* cells' characteristics, host and/or abiotic surface properties, such as cell-surface hydrophobicity and cell wall composition [30,81]. The *C. glabrata* cell wall is the site for physicochemical interactions between the microorganism and the surfaces, leading to its adherence. Although the lack of studies concerning this issue, it is assumed that the cell surface of *C. glabrata* cells reportedly exhibits a degree of hydrophobicity comparable with *C. albicans* [82]. Interestingly, however, while the hydrophobicity of *C. albicans* has showed to be extremely sensitive to specific growth conditions, numerous isolates of *C. glabrata* were relatively insensitive to those same growth conditions [83]. Similarly to *C. albicans*, *C. glabrata* adhesion phenomenon is mediated by specific proteins on its cell wall - epithelial adhesins (*Epa*) - that have a comparable structure to the Als (Agglutinin like sequence) proteins [84]. The *EPA* genes family are composed of 17-23 genes depending on the strain, though *EPA1*, *EPA6* and *EPA7* are the most important [85]. The overall structure of Epa proteins is similar to that of the Als proteins of *C. albicans*. Although, there are few studies concerning *C. glabrata* Epa proteins, it is known that Epa1 is a Ca^{2+} dependent lectin that binds to N–acetyl lactosamine–containing glycolcoconjugates [84]. Furthermore, despite the large number of *EPA* genes, it has been shown that the deletion of merely *EPA1* reduces the *in vitro* adhesion to host epithelial cells [86] and this adhesin is inhibited in the presence of lactose [87]. It was indicated that *C. glabrata* strains are unable to express *EPA6 in vitro*, however a report demonstrated that it was expressed during urinary infection, due to low levels of nicotinic acid, suggesting that *C. glabrata* is capable to adapt to different environmental conditions [87,88]. *EPA6* and its close paralogue *EPA7* are located in subtelomeric regions and their transcription is regulated by Sir4 and Rif1, two proteins involved in subtelomeric silencing. Biofilm growth conditions induce the transcription of *EPA6* and *EPA7*: this is dependent on the presence of an intact subtelomeric silencing machinery and is independent of the Mpk1 signalling pathway. Finally, the kinase Yak1 is required for expression of both adhesins' genes and acts through a subtelomeric

silencing machinery-dependent pathway [80]. A study made to identify and describe genes involved in the formation of biofilms by *C. glabrata* recognized the Cst6 transcription factor as a negative regulator of the *EPA6* gene, which encodes an adhesin central to *C. glabrata* biofilm development [89]. Analysis of mutant strains disclosed that Cst6 acts in a pathway independent of the Yak1/Sir4 pathway, also known to regulate expression of *EPA6* and, consequently, biofilm formation. In contrast, the chromatin remodelling Swi/Snf complex, which is adhesin-specific, positively regulates biofilm formation in *C. glabrata*. Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) experiments demonstrated that *EPA6* expression depends on the integrity of the Sir complex [89].

Groot et al [90] identified another family of adhesins involved in the first stage of *C. glabrata* biofilm development, namely Awp adhesins. Initially, four Awp adhesins (Awp1-4) were identified using liquid chromatography tandem mass spectrometry (LC-MS) [90] and a subsequent study revealed the gene expression profile of the seven Awp adhesins (Awp1-7) [91]. The initial attachment of *C. glabrata* cells is followed by cell division and this proliferation leads to the formation of a basal layer of anchoring microcolonies, with following biofilm maturation [30,79]. Biofilm conditions and high cell density are adhesion inducers, activating *EPA6*, whereas *EPA1* is triggered typically in the lag phase and the *C. glabrata* biofilm maturation is characterized by the production of extracellular matrix [30,92].

Very recently, Chupácová et al [93] reported the results of a study with a new protein involved in adherence and biofilm development. The CR3-RP (complement receptor 3-related protein) is one of the surface antigens expressed by *Candida* spp.. The authors studied the effect of a polyclonal anti-CR3-RP antibody (Ab) on the adherence and the biofilm formed by *C. albicans* SC5314 and *C. dubliniensis* CBS 7987 and two clinical isolates. After pre-treatment with the anti-CR3-RP Ab, a 22-41% reduction in adherence with a decreased thickness in biofilms formed by both species was noticed. The data suggest a potential activity of the anti-CR3-RP Ab relevant to immunotherapy or vaccine development against biofilm-associated *Candida* spp. infections, including *C. glabrata*.

I.1.4.2 Biofilm formation

Besides possibly being a key factor for the survival of *C. glabrata*, biofilm formation may also be responsible for the particularly well adapted colonization of tissues and indwelling medical devices [94]. Comparing with the other *Candida* spp., *C. glabrata* displays the lowest biofilm metabolic activity although having the highest number of biofilm cultivable cells [95]. Likewise, the metabolic

activity of resuspended *C. glabrata* biofilm and planktonic cells is lower than that of the other species [95]. Thus, generally, *C. glabrata* biofilms have less total biomass and its strains show similar biofilm forming ability, in opposition to *C. parapsilosis* and *C. tropicalis. Candida glabrata* biofilms often display a structure of a multilayer with blastoconidia intimately packed or constituted by clusters of cells with, in both cases, total absence of pseudohyphae and hyphae. Remarkably, biofilm matrices of *C. glabrata* have relatively higher quantities of carbohydrate (in some cases five times more quantity) and protein, compared with the other NCAC spp. [30,88,95].

Biofilm conditions and high cell density are adhesion inducers, activating *EPA6*, whereas *EPA1* is triggered typically in the lag phase [80,84]. This complex regulation of adhesin genes in *C. glabrata* is reminiscent of the complicated regulation of *S. cerevisiae* flocculation (*FLO*) genes. Indeed, *FLO11* is one of the genes regulated by the Swi/Snf complex [89,96]. Thus, reliable with the knowledge that adhesion of *C. glabrata* to host epithelial tissue plays an important role in virulence, the genes *FLO1*, *FLO5*, and *FLO10* were studied in *S. cerevisiae* and were found to be Pdr1-dependent [97]. In *C. glabrata FLO10* (named *EPA1*) is essential for effective *in vitro* adherence to human epithelial cells, evidencing a role for *EPA1* in adherence to abiotic materials [98]. *FLO5* has been described as a member of the EPA family of adhesion proteins in *C. glabrata*, however, its role in adherence has not been completely studied. The link between *FLO* and *EPA* genes prove that its regulation may control the adherence of *Candida* spp. during the host-interactions, conferring distinct adhesion profiles toward human proteins and cells [99,100]. This regulation is variable, among species and even between strains of the same species [100].

The main genes and proteins linked to virulence in *C. glabrata* are summarized in Table I.2.

I.1.4.3 Enzyme production

The ability to destruct host tissues by *Candida* spp. is a strong virulent factor that, is facilitated by the release of hydrolytic enzymes into the local environment. The general secretion of enzymes, such as, proteases, phospholipases, lipases and haemolysins, help the yeasts to survive and replicate within macrophages, being able to destroy the epithelium. As referred, *C. glabrata* do not produce proteases, in opposition to the rest of *Candida* spp. [95,98]. However, there is one study that has shown that this species is capable of proteinase production, but the type of proteinase was not specified [101].

Phospholipases (PLs) hydrolyze phospholipids into fatty acids and its production contribute to host cell membrane damage, promoting cell damage or exposition of receptors that facilitate adherence [102].

Table I.2. Genes and/or proteins associated to *Candida glabrata* virulence

Function	Genes/Proteins Involved	Reference(s)
ABC transporters	*PDR1, CDR1, CDR2, CDR3 CDR4, SNQ2* and *MDR* family	[103–116]
Adhesion	*EPA* family, *SIR3* and *RIF1*	[80,84,89,98,117–125]
Adhesion and Colonization	*YPS* family	[126,127]
β-1,3-glucan biosynthesis	*FKS1, FKS2, XOG1*	[85,105,107,113,128–144]
β-1,6-glucan biosynthesis	*KRE1, KRE9, SKN1, KHN1, XOG1*	[107,118,144–146]
Biofilm formation	*BCR1, SIR4, RIF1, YAK1*	[80,147,148]
Extracellular matrix regulators	*ZAP1, GCAL1, ADH5, CSH1*	[107,149,150]
Ergosterol biosynthesis	*ERG* family	[107,151–154]
General virulence	*ACE2*	[140,152,155–160]
Heat shock processes	*HSP* family	[107,161,162]
Mitogen activated protein kinase (MAPK)	*MCK1*	[107,163]
Major facilitator family (MFS) transporters	*MDR1, FLR1*	[107,114,115,129]
Oxidative stress processes	*CTA, YAP1,* Msn2, Msn4 and Skn7	[164–172]
Phagocytosis survival	*ATG11, ATG17, Yps*	[122,165]
Pigmentation Oxidative stress processes	*ARO*	[173]

Candida glabrata produces phospholipases, which promotes a greater and powerful interface with the host mucosae, destroying it and facilitating an effective invasion of the tissues involved [102]. Lipases are involved in the hydrolysis of triacylglycerols, which are also produced by *C. glabrata*. In *C. albicans*, 10 genes encoding for lipases have been identified and it has been shown that mutants were significantly less virulent in a murine intravenous infection model. Sequences similar to *C. albicans* (LIP1-10) were also detected in *C. tropicalis*, but not in *C. glabrata* [102,174–177]. *C. glabrata* has demonstrated to have capacity to degrade hemoglobin-using haemolysins in order to obtain iron [178,179]. Alike the others *Candida* spp., *C. glabrata* can grow in the host by degrading hemoglobin and extracting the elemental iron from host cells for metabolic processes. Although haemolysins are known to be putative virulence factors backing pathogenicity in *Candida* spp., the genetic expression of haemolytic activity of *C. glabrata* is still poorly understood. Luo et al [180] revealed that a haemolysin-like protein gene (*HLP*) was related with the haemolytic activity of *C. glabrata*. The assembly of the haemolytic factor may be regulated by the presence of glucose

in the growth medium. *Candida glabrata* and others NCAC spp. are able to produce haemolysins *in vitro*, bringing partial or total erythrocyte lyses, though the extent of this is both strain and species dependent [180,181].

I.1.5 Candida glabrata-*Host interactions*

Candida glabrata cells are able to commensally colonize the mouth, esophagus, intestines and vagina mucosal surfaces, but very little is still known about its interaction with the host and defense mechanisms. Still, the high prevalence of *C. glabrata* infections in immunocompromised individuals indicates that some level of host defense does indeed exist. Moreover, normal host mechanisms are expected to control *C. glabrata*, holding it in check and suppressing the expression of its pathogenic properties, preventing the infection [182,183].

Phagocytic cells (e.g. neutrophils, dendritic cells and macrophages) constitute the primary response of the innate host immune system against *Candida* spp. infections. After internalization of the fungal pathogen, formation of the phagolysosome continues inside the phagocytic cells, for the destruction of the microorganism. The organelle harbors a damaging and highly toxic internal phagolysosome for the elimination, including low pH, hydrolytic enzymes, potent reactive oxygen and nitrogen species (DNA damage), proteins and lipids of the pathogen [183–185]. Trapped microorganisms have developed diverse strategies to survive the oxidative burst and to escape from the phagolysosome, including many antioxidant defense enzymes (e.g. catalase, reductase, superoxide dismutase, thioredoxin- and glutathione-dependent peroxidases) [183–185]. *Candida albicans* and *C. glabrata* carry only one catalase gene [186,187]. Since both stress and non-fermentable carbon source can induce expression of *CTA1,* the regulation of the catalase gene is a combination between two catalase genes which can be found in *S. cerevisiae* [165]. Though not yet unswervingly shown, the complex regulation and conservation of this enzyme in *C. glabrata* suggests a role for peroxide stress resistance in the host environment as well [165].

In this context, after the attachment, in a *Candida* spp. infection, the ensuing step in the pathogenesis process is invasion, normally into epithelial cell layer, but, *in vivo* invasion probably relies on endocytosis, and with *C. glabrata*, instead of active penetration, with poor host cell damage and low pro-inflammatory response [188–190]. Although it is recognized that endothelial cells can phagocytize *C. albicans*, with *C. glabrata* this is not induced, which suggests that this endothelial-cell activity may be species specific or restricted to *C. albicans* alone [191]. As seen, macrophages and neutrophils are part of the first line of host immune defence when *Candida* spp.

cells infect the bloodstream or the endothelia [192–194]. Conversely, both conventional and unconventional immune cells could play some role in innate and/or acquired host defence against *C. glabrata* infection [19]. The B cells are known to mediate host resistance to intravenous (i.v). established *C. albicans* systemic infection [195], but in *C. glabrata,* the B cells stimuli is reported not to be so relevant [188,196]. On contrary to a strong neutrophil infiltration - characteristic for *C. albicans* infections -, *C. glabrata* either does not stimulate or is able to suppress neutrophil attraction and is slightly related with mononuclear cells [188,196]. Indeed, the existence of hyphae and occurrence of host cell damage in *C. albicans* infections leads to a stronger pro-inflammatory cytokine response than in *C. glabrata* infections [27]. Additionally, authors have shown that *C. glabrata* is recognized and ingested by macrophages at a much higher rate than *C. albicans* [197]. It is documented that, after recognizing pathogens, macrophages release cytokines that help coordinate the immune responses, but, when *C. glabrata* is internalized by macrophages, it alters the normal phagosome maturation process [198], surviving through autophagy and replicating inside the phagosome until the bursting of the phagocyte [122,165,198]. To escape the host defense mechanisms, variations of the morphology of the microorganisms allows the burst from phagocytotic cells. *Candida glabrata* is capable to detoxify radical oxidative species and seems to disrupt normal phagosomal maturation, leading to inhibition of phagolysosome formation and phagosome acidification [198]. In terms of starvation, *C. glabrata* autophagy is used for intracellular nutrient mobilization, being vital for its virulence. It is a highly controlled process and needs a number of factors for the correct recognition, packaging and delivery of cargo to the vacuole [199–201]. *Candida glabrata* survives the defense mechanisms induced by the macrophage, including largely oxidative stress.

In yeasts, a great majority of the almost 500 stress genes are under the control of four transcription factors: ScYap1 (the central component of the oxidative stress response regulated by H_2O_2 or diamine), ScSkn7 (important for peroxide stress protection and for the induction of *TRX2*, *TRR1*, *TSA1* and *CTA1* [202]) and ScMsn2/ScMsn4 [203,204]. The core response to oxidative stress consists of thioredoxin cofactor Trx2, glutathione peroxidase Gpx2, catalase Cta1, thioredoxin peroxidases (Tsa1 and Tsa2) and thioredoxin reductases (Trr1 and Trr2*).* Finally, the transcription of the *C. glabrata* copper–zinc superoxide dismutase (SOD) genes *SOD1* and *SOD2* is regulated in another way from yeast. Both yeast *SODs* are induced by carbon source depletion, whereas, significantly, *SOD1* is expressed constitutively [205].

While inside the macrophage, fungal pathogens control their metabolism to the glucose-deficient environment and adjust their expression profile towards genes involved in alternative carbon sources [122]. Genes associated to the glycolytic pathway are repressed, whereas genes encoding for proteins needed for gluconeogenesis, β-oxidation, glyoxylate cycle, autophagy, peroxisome proliferation, and amino and acetate transports are induced [122,206]. Snf1, a protein kinase, stimulates the transcription of glucose-repressed genes, playing a central role during glucose starvation in *S. cerevisiae* and *C. glabrata*. The presence and absence of glucose leads to similar changes of transcription in *C. glabrata* as in *S. cerevisiae* [170].

Host–pathogen interaction demands numerous crucial functions achieved by proteins of the cell wall, such as tissue adhesion, invasion and biofilm formation, protection from host defense mechanisms, triggering of the host immune system and resistance to antifungal drugs [207]. *Candida glabrata* adherence to the host epithelial tissue is mainly mediated by a number of glycosylphosphatidylinositol (GPI)-linked adhesin genes [86,208], some induced in *C. glabrata* cells from biofilms [91]. As already discussed, its genome (ATCC2001) covers 67 genes encoding putative adhesin-like GPI-modified cell wall proteins including the Epa family [90] and their expression is well regulated. *C. glabrata* cells carrying mutations in some loci show an increased adherence to epithelial cells and are able to colonize organs more efficiently [209–211]. In fact, there are several factors related to the development of *C. glabrata* infection. Naturally, these features promote the pathogenicity performing together and depend on the species characteristics itself and the host physiology conditions, specifically the state of its immune system [211]. A model using a reconstituted human oral epithelium (RHOE) infected only with *C. glabrata*, or simultaneously with *C. glabrata* and *C. albicans,* showed that *C. glabrata* strains originating from the oral, vaginal and urinary tract were able to colonize the tissue but in a strain dependent manner. Single infection with *C. glabrata* showed no invasion of the RHOE but mixed infection showed that *C. albicans* enhanced the invasiveness of *C. glabrata* [212]. The results obtained by these authors demonstrated an enhanced invasion and increased tissue damage caused by mixed *C. glabrata* and *C. albicans* infections, what has important clinical significance and highlights the need to identify *Candida* spp. involved in oral candidiasis. Jayatilake et al [88] also used a RHOE model to study host-interaction and the virulence factors in oral candidiasis induced by *C. albicans* and NCAC spp., using mutated species. All *C. albicans* isolates invaded the tissue over 48 h. The invasive potential of hyphal and *SAP* (secreted aspartyl proteinases) mutants was similar to the parent wild-type isolate at 12 h, although after 24 h their invasion was dissimilar. NCAC spp. and

hyphal mutants were all non-invasive and the second most pathogenic group comprised C*. albicans* with *C. glabrata*. This study supports the theory that the conversion from the blastospore phase to the hyphal phase is an important virulence attribute of *C. albicans*, showing also that for the NCAC spp. (which include *C. glabrata*), is necessary to focus in other features to understand and to contour NCAC spp. infections.

Brunke et al [22] showed that a continuous co-incubation of *C. glabrata* with a murine macrophage cell line for over six months resulted in the adjustment in fungal morphology to pseudohyphae, which was stable over several generations without any selective pressure and supplemented by changes in cell wall architecture. The pseudohyphae-like structures allowed a faster escape from macrophages and amplified the damage of macrophages. In addition, the evolved strain (Evo) exhibited a momentarily increased virulence in a systemic mouse infection model, which was associated with an increased organ-specific fungal burden and inflammatory response (TNF-α and IL-6) in the brain. The genetic analyses of the Evo strain exposed a single nucleotide exchange in the chitin synthase-encoding *CHS2* gene as the single basis for this phenotypic alteration. A targeted *CHS2* mutant with the same single nucleotide polymorphism (SNP) showed similar phenotypes as the Evo strain under all experimental conditions tested. The fallouts signpost that microevolutionary processes in host-simulative conditions can induce adaptations of *C. glabrata* to distinct host niches and lead to the appearance of hypervirulent strains [22].

I.2. Biofilms of *Candida glabrata* and the associated resistance mechanisms

Fungi in general, and *Candida* spp. in particular, are non-motile microorganisms. The biofilm structure, thus, reflects the sequence of cell division events that occur during a biofilm development and results in an exceptionally resistant profile of the biofilm cells to one or several antifungal drugs classes. Biofilm infections are complicated due to the presence of robust inducible gene networks encoding different proteins that confer tolerance or resistance to antifungal drugs [213]. The antifungal resistance mechanisms can be classified as either microbiological or clinical [214]. The first are defined by the presence of a developed or mutational resistance mechanism to a specific drug. It depends directly on the microorganism and it is distributed into two groups: primary or innate in which fungi are resistant prior to drug exposure and secondary or acquired when it appears in response to a drug exposure. Clinical resistance is the result of a failure in the infection treatment [214]. Regarding to susceptibility or resistance, a *Candida* spp. are defined as susceptible or resistant by the level of antifungal drug activity associated with a high likelihood of

therapeutic success or therapeutic failure, correspondingly. Within the resistance concept, a *Candida* strain can be defined as intermediate by a level of antifungal activity associated with uncertain therapeutic effect. Resistant is when it indicates growth or lack of inhibition with persistence of fungal infection, with correct antifungal drug therapy [215]. Thus, *Candida* spp. are categorized as susceptible, intermediate or resistant, using defined breakpoints by an appropriate antifungal phenotypic test system. Several general mechanisms of biofilm drug resistance are thought to confer resistance to multiple classes of antifungals in *Candida* spp. biofilms: the up-regulation of efflux pumps, the cell wall composition, the increased cell density and *quorum sensing* effect, the presence of an extracellular matrix, the changes in metabolism, the presence of persister cells, the cellular signalling and the stress responses [213,216–219].

Table I.3 summarizes of the most relevant factors involved in *C. glabrata* antifungal resistance.

I.2.1 Up-regulation of efflux pumps and cell wall composition

Two main classes of efflux pumps contribute to antifungal drug resistance: the ATP binding cassette (*ABC*) transporter superfamily containing *CDR1* and *CDR2*, and the MFS containing *MDR1* [213,220,221]. While under treatment with antifungals, biofilm cells up-regulate these transporters within six hours of surface contact both *in vitro* and *in vivo*, even in the absence of drug [222–225]. In 2013, Fonseca et al [78] described that the usual *ABC* transporters were upregulated in biofilms of three *C. glabrata* strains. Similarly, another *ABC* transporter, *PDR1*, was evaluated and was found to be overexpressed. These alterations were allied to modifications in the structure of *C. glabrata* biofilms by creating cell clusters, which could be a possible mechanism of biofilm tolerance to fluconazole. The surface adherence only, has showed to be enough to intensify the expression of the genes encoding the efflux pumps [226] which are also up-regulated in mature biofilms, demonstrating that they continue to mediate drug resistance throughout biofilm development [227–229]. Taff et al [144] studied mutants related with enzymes encoded by *BGL2*, *PHR1*, and *XOG1*, and reached the conclusion that they did not appear to disturb cell wall glucan composition of biofilm cells, nor were they necessary for filamentation or biofilm formation.

Although and curiously, the results revealed that mutants missing these genes demonstrated developed susceptibility to fluconazole, only during biofilm growth. Moreover, results in transcriptional analysis and biofilm phenotypes of strains with multiple mutations propose the studied enzymes perform in a complementary manner in order to distribute matrix downstream of the primary β-1,3-glucan synthase encoded by *FKS1*.

I.2.2 Increased cell density and quorum sensing

It is well recognized that the inoculum size can affect susceptibility results [213,230–233]. Thus, using the microtiter method to test *Candida* spp. for drug susceptibility, an optimal inoculum size range was defined as a clinical standard. Indeed, if this cell concentration is augmented in the drug resistance assays, resistance specifically to fluconazole, ketoconazole, amphotericin B and caspofungin is increased up to twenty-fold [233].

Table I.3. Summary of the most relevant factors involved in *Candida glabrata* antifungal resistance

Factor	Description	Reference(s)
Tolerance	Pathogenic fungi encounter and bear a range of physiological stresses from different environments: ionic stress, changes in osmolarity, and oxidative stress (e.g. phagosomes of neutrophils).	[234–238]
Environmental stress	pH, temperature, oxygen availability, nutrients availability These factors, associated to inter-relationships with other yeasts and bacteria, creates multiple permutations of strain specific biofilms, exhibiting distinct and unique biofilm fingerprints	[236,239–246]
Cell Density	Mature biofilms, whilst densely populated, exhibit spatial heterogeneity	[235,236,239,247,248]
Efflux-Pump-Mediated Resistance	Exocytosis of the drug	[224,228,249–254]
Extracellular Matrix	Presence of extracellular matrix is an essential characteristic of fungal biofilms, providing the cells protection from hostile factors and immune evasion	[92,235,236,255–258]
Genetic alterations	Overexpression of genes related with drug targets	[213,216,234,255,259,260]
Persister cells	These cells are “dormant variants of regular cells that form stochastically in microbial populations and are highly tolerant to antibiotics”. They are a vital mechanism of resistance in chronic infections.	[213,261–266]

By contrary, if biofilms are dissociated and analysed at a lower density in the same assays, they show drug susceptibilities at the level of planktonic cells evaluated at the same cellular density [233]. In another phenomenon within the biofilm environment, *Candida* spp., cells have the ability

to communicate with each other by *quorum sensing via* numerous signalling molecules which is directly dependent on the cell density [267].

I.2.3 Extracellular matrix

In *Candida* spp. biofilms, carbohydrates, proteins, and nucleic acids form the extracellular matrix that surround the cells in the biofilms [268–272]. It is thought that biofilms prevent, in more or less extent, the penetration of antifungal drugs through their structure, by the establishment of a diffusion barrier, as an ion-exchange resin, binding charged antibiotic molecules, contributing to biofilm drug resistance [79,269,273]. The most important are the β-glucans, which are polymers of the fungal cell wall and are a substantial constituent of the biofilm *Candida* spp. matrix. When induced, the disruption of β-1,3-glucans or a β-1,3-glucanase treatment has shown to increase susceptibility of biofilms to fluconazole and an addition of exogenous β -1,3-glucans has demonstrated to rise resistance to fluconazole in planktonic cells [274]. Also, it is possible that biofilms can also sequester amphotericin B as it has been presented that β-1,3-glucans can bind specifically to this drug [79,275]. An induced compromised expression of the *FKS1* gene has revealed to improve the effectiveness of anidulafungin, flucytosine and also amphotericin B [276]. It is known that planktonic cells generally rely on irreversible genetic changes to maintain a resistant phenotype, while biofilms are able to persist due to their physical presence and the density of the population, which affords an almost inducible resistant phenotype notwithstanding of distinct genetic alterations [213]. The application of DNA microarray and proteomic technologies can facilitate a more detailed analysis of the biofilm lifestyle [117,277]. Specific biofilm formation genes are being brought up regarding different roles in biofilm resistance: peroxisomal catalase (*CTA1*), the biosynthesis and degradation of tyrosine genes (*ARO*), the muscle creatine kinase (*MSK*), the heat shock protein 90 (*HSP 90*), the sphingolipid biosynthesis (*SKN 1* and *KRE1*), *SIR*, *RIF* and, finally, the extracellular matrix (ECM) regulators: zinc regulated *(ZAP1)*, g-carbonic anhydrase (*GCAL1*), alcohol dehydrogenase (*ADH5*) and also cell surface hydrophobicity (*CSH1*) [213,277].

I.2.4 Metabolism and stress response

Alterations in temperature or in specific nutrients, restricted nutrient availability, ionic stress, variations in osmolality and oxidative stress are all acknowledged as sources of resistance mechanisms of biofilms. A study [239] showed that the resistance to chlorhexidine, fluconazole, amphotericin B and nystatin increased as biofilms mature over time, corresponding to a growth in metabolic activity over biofilm maturation. Though, in these experiments, cell number was not

controlled, being unclear if this is a true demonstration of metabolic activity [239]. Also, in developing *C. albicans* biofilms, the Krebs cycle is down-regulated over time but it is unknown if it is linked to drug resistance [229,278]. Baillie and Douglas [279] have shown that, at lower growth rates, planktonic cells are more resistant to amphotericin B, and biofilms are equally resistant over a range of growth rates, proposing that growth rate plays only a minor role in *Candida* spp. biofilm drug resistance. In other study [280], the same authors demonstrated that neither glucose nor iron limitation disturb *Candida* spp. biofilm resistance to amphotericin B. However, iron limitation increased the susceptibility of dispersed daughter cells from biofilms to amphotericin B, detected by a number of cells which induced responses by signaling pathways [234]. Seneviratne et al [281] showed that there is a positive regulatory protein of the stress response in biofilms of stressed *C. glabrata*, displayed by the heat shock and other stress proteins (Hsp12, Trx1 and Pep4).

I.2.5 Persister cells

Persister cells form, randomly and inside the biofilm cell population, an unique group, phenotypically dormant, highly resistant to antifungal drugs [262] and a key mechanism of resistance in chronic infections [213]. Yeast persister cells were first discovered as a small population in *C. albicans* biofilms [262,282]. These cells were extremely drug resistant, independently of drug efflux pumps and the composition of the cell membrane. *Candida* spp. persister cells are exclusively recovered from biofilms and not from planktonic populations, notwithstanding of their growth phase, and involve the attachment to a substrate to initiate the dormant phenotype. These cells are believed to be a phenotypic variant of the wild type strain, for they are result of a biofilm with new subpopulations [219]. LaFleur [262] showed that, when biofilms were treated with amphotericin B or chlorhexidine, most cells were eliminated, but a subpopulation of highly tolerant sessile cells was persevered. Later, Lewis [283] also indicated the same results [266,284]. In clinical isolates of *C. albicans*, persister cells are found at an expressively higher incidence from patients with long-lasting *Candida* spp. infections, when compared to patients with temporary infections. Other report[261] indicated the presence of persister cells in biofilms treated with amphotericin B from isolates of *Candida* spp.. Bojsen and collegues [285] performed a study in order to evaluate whether resistance mechanisms on amphotericin B was shared between biofilm and planktonic populations. A multiplexed barcode sequencing screening of a combined group of gene-deletion mutants cultivated as biofilm and planktonic cells associated to an assay for resistance to the ergosterol-targeting fungicide

amphotericin B was executed. The results revealed that the biofilm and planktonic had substantial overlap in amphotericin B-persistent mutants. Also, the authors could demonstrate that the mutants had defective sterol metabolism, ribosome biosynthesis, and in the *TORC1* (ubiquitin binding activity, role in cellular response to starvation, regulation of cell growth) and in the Ras pathways (protein signal transduction), displayed an amplified persistence when treated with amphotericinB. The *ras1*, *ras2* and *tor1* mutants had a high-persister phenotype comparable to wild-type biofilm and planktonic cells exposed to the *TORC1* pathway inhibitor rapamycin, and, in the other side, the inhibition of *TORC1* with rapamycin similarly improved the proportion of persisters in *C. glabrata.* With these results, the authors have demonstrated that a decreased *TORC1*-mediated induction of ribosome biosynthesis via Ras can originate the development of amphotericin B-persister cells in planktonic, but also in biofilms [285].

I.3. General mechanisms of antifungal drug resistance

The antifungal resistance is both complex and multifaceted. It can be originated in response to a compound, or an irreversible genetic change resulting from prolonged exposure. Alterations or overexpression of target molecules, active extrusion through efflux pumps, limited diffusion, tolerance, and cell density, are all characterized mechanisms utilized by fungi to combat the effects of antifungal treatments [286]. Planktonic cells generally rely on irreversible genetic changes to maintain a resistant phenotype, whereas biofilm cells are able to persist due to the persistence and density of the population, which provides an almost inducible resistant phenotype irrespective of defined genetic alterations [286].

In the clinical practice, resistance is the result of a failure in the infection treatment [214]. *Candida* spp. are defined as susceptible or resistant by the level of antifungal drug activity associated, correspondingly, with a high likelihood of therapeutic success or therapeutic failure. There has been an epidemiological change from *C. albicans* to NCAC spp., with *C. glabrata* and *C. krusei* emerging as important and potentially antifungal resistant causes of candidaemia [287]. Notably, *C. glabrata* has been frequently reported as exhibiting variable karyotypes between isolates [288–295], and several studies with *Candida* spp. have demonstrated that these karyotypes are relatively stable, conjecturing that the karyotype of virulent species is more stable than the avirulent ones [296]. The major karyotypic differences between *C. glabrata* strains are associated to a small number of chromosomal translocations. Along with variation in the subtelomeric *EPA* genes, the

other genomic rearrangements are copied number variation in tandem gene repeats, encoding putative or known cell wall proteins [289].

Bader et al [297] analyzed the derivatives in *C. glabrata* strains' genome, which were shown to be indistinguishable by multi locus sequence typing. Yet, divergent phenotypic groups that were related to specific karyotypic changes were also spotted. Chromosomal aberrations and functional adaptations can occur during infection and under antimicrobial therapy, but also under laboratory conditions deprived of extreme selective pressures and can significantly affect phenotypic properties (e.g. the cell wall carbohydrate composition and quantitative changes in adhesion genes expression), being noticed slightly than subtelomeric genes loss or differences in the number of macrosatellite repeats within adhesion genes. Chromatin alterations can also occur, as essential strategies of survival, which simplify a reprogramming of cellular energy metabolism in macrophage-internalized *C. glabrata* cells, and provide protection against DNA damage [298]. Thus, as all *Candida* spp., *C. glabrata* has the competence to riposte to the environmental alterations aimed to adapt to the presence of antifungal agents and in this way provide protection against antimicrobial therapies [211].

Cell wall fluctuations, but mostly immunoevasion and intracellular persistence, may be the crucial factors in the ability of *C. glabrata* to persist in the course of multiple antifungal treatments and to develop multidrug resistance [80,89]. Consequently, different mechanisms of resistance vary among drugs typically due to the mode of action of each class of antifungal. There are, presently, three main antifungal classes: azoles, polyenes and echinocandins. The azoles are known to have fungistatic activity, targeting the ergosterol biosynthetic pathway, by binding to the Cyp51 family of cytochrome P450 - the 14-α-lanosterol demethylases - that are encoded by the *ERG11* gene. These drugs are responsible for the lack in the capacity to build and renew sterols in the cellular membranes, changing membrane fluidity and function of vital processes such as signaling, transport, exocytosis and endocytosis [44]. Fluconazole, voriconazole, itraconazole, clotrimazole, miconazole and posaconazole are main examples. The polyenes (e.g. amphotericin B, nystatin) and echinocandins (e.g. micafungin, caspofungin, anidulafungin) are fungicidal agents. The first ones bind to the ergosterol of the fungal cell membrane establishing transmembrane aggregates pores, which causes membrane depolarization with subsequent increase its permeability to monovalent protons and cations. This allows the passage of intracellular molecules the external environment, initiating an osmotic imbalance and, finally, cell death [299–301]. Echinocandins interfere with the fungal cell wall synthesis through a non-competitive inhibition of β-1,3-glucan

synthesis [302], which results in weakening of the cell wall, breakdown of cellular integrity and cell lysis [303].

Other less used antifungals comprise the aantimetabolites, such as 5-fluorocytosine, which inhibits RNA function and, to close, the allilamines, morpholines and a chitin synthase inhibitor, as the nikkomycin. Figure I.3 synthetizes this information.

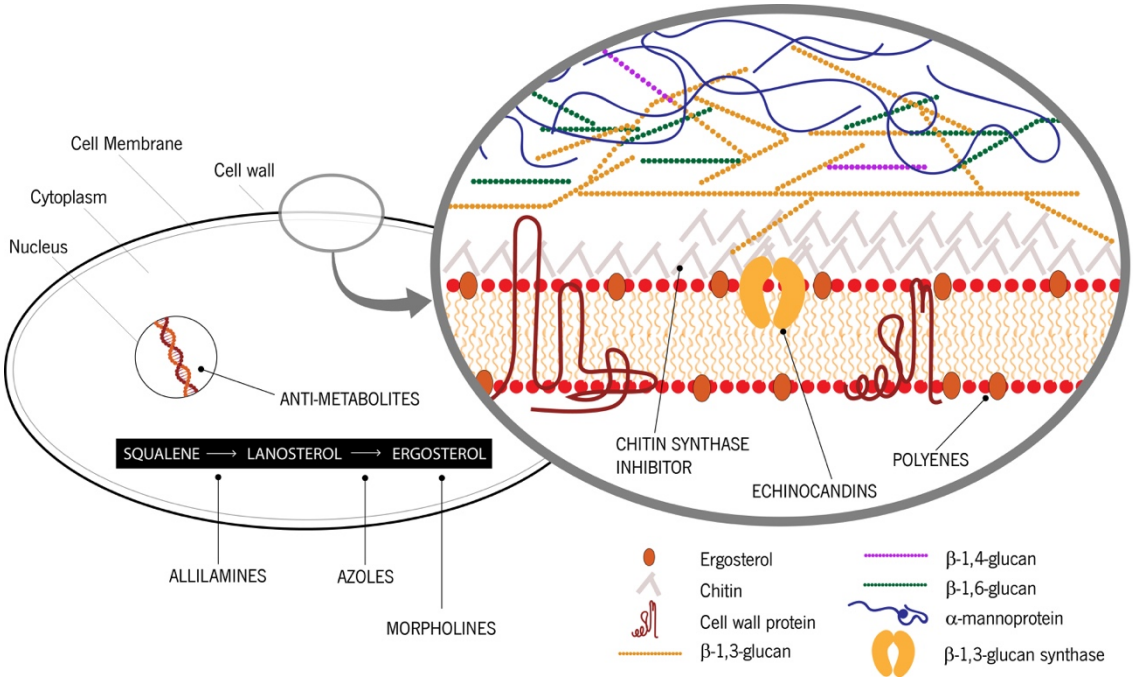


Figure I.3. Mechanisms of action of the classes of antifungal drugs.

The mechanisms of antifungal resistance are categorized as primary or secondary and are related to intrinsic or acquired characteristics of the fungal pathogen, including the interference with the antifungal mechanism of the respective drug/drug class or the decrease in target drug levels [54]. Genomic plasticity is an important machinery for adaptation to environmental signs, such as host responses and antifungal drug pressure in many fungi, including *C. glabrata*. As an example, *C. glabrata* genome has lost genes involved in galactose and sucrose assimilation, phosphate, nitrogen and sulfur metabolism, as well as thiamine, pyridoxine and nicotinic acid biosynthesis [36,54]

The dynamics of efflux phenomena from procaryotic or eucaryotic cells strongly modulates the activity of a large number of antifungals. Inside the group of primary active transporters, predominant in eucaryotes, six families belonging to the ATP-binding cassette superfamily, and including the P-glycoprotein in the *MDR* (Multi Drug Resistance) group and the *MRP* (Multidrug

Resistance Protein), have been recognized as being responsible for antibiotic efflux [216,304]. Within the class of secondary active transporters (antiports, symports, and uniports), five families of antibiotic efflux pumps have been described, distributed in five superfamilies [*SMR* (Small Multidrug Resistance), *MAR* (Multi Antimicrobial Resistance), *RND* (Resistance Nodulation Division), and *MFS* (Major Facilitator Superfamily)]. In eucaryotes, the antibiotic efflux pumps modulate the accumulation of antimicrobials in phagocytic cells and play a major role in their transepithelial transport [304]. These and other factors will be next explored.

I.3.1 Resistance to azoles

During the past decades there was a remarkable increase in mucosal diseases caused by *Candida* spp.. These infections were due to the increase of immunosuppressive diseases (e.g. cancer, AIDS) associated with an extraordinary emergence of resistance to azoles [305]. In the early 1990s, fluconazole became the first-choice drug in the treatment and prophylaxis of oro-oesophageal candidiasis. Subsequently, in the following years, the resistance was described in up to 41% of the patients [306–308]. Also, an increase in cases of fungemia caused by NCAC spp., specially *C. glabrata* and *C. krusei* [56,309–311]. As recognized, *C. glabrata* grows only as a yeast form *in vivo* and its adhesion is relatively weak, when compared to *C. albicans* [304,312,313]; thus, it is believed that the increase of *C. glabrata* infections is associated to that same inherent low susceptibility to azoles [314] and that the acquired resistance outcomes from rare mutations that are selected by drug pressure [154].

The acquired resistance of *C. glabrata* to azoles is connected to several mechanisms, but the most common is the induction of efflux pumps, encoded by the *ABC* transporter genes (*CDR1* and *CDR2*, *SNQ2*) or to *MDR* belonging to the MFS that lead to decreased drug concentrations [316]. In *C. glabrata*, the transcription factor Pdr1 is involved in resistance to azoles through upregulation of *CDR1*, *CDR2* and *SNQ2* [128]. The mitochondrial dysfunction associated to the development of the "petite mutants", which have mitochondrial DNA deficiency and upregulate the *ABC* transporter genes, highly amplifies the resistance to azoles, leading to a drastic improvement of the *C. glabrata* antifungal resistance [317]. A number of *ABC* transporters, including Cdr1, Pdh1 (also known as *CDR2*), Yor1, and Snq2, contribute to xenobiotic efflux phenomena. The transcription factor Pdr1 is the main regulator of *ABC* transporters genes expression and the key component of Pleiotropic Drug Resistance (*PDR*) [103,108]. In *C. glabrata*, Pdr1 forms a heterodimer with Stb5 in *S. cerevisiae* and transcriptional analysis pointed out a shared region among the homologues of these

two genes, *PDR1* and *STB5*, and many of the genes upregulated by overexpression of *PDR1* were upregulated by deletion of *STB5.* Accordingly, the *PDR1* overexpression and *STB5* deletion are correlated [105,147]. It was found that the overexpression of *STB5* in *C. glabrata* represses azole resistance, while its deletion produces a minor intensification in resistance. Expression analysis assays recognized that *STB5* shares many transcriptional targets with *PDR1* but, unlike the second, it is a negative regulator of pleiotropic drug resistance (including the *ABC* transporter genes *CDR1, PDH1*, and *YOR1*) [105,128]. A Farahyar et al [318] study demonstrated that *CDR1* and *CDR2* genes are expectedly upregulated in azole-resistant isolates (≥2-fold) and that, the fatty acid activator 1 (*FAA1*) gene presented a ≥2-fold expression in resistant isolates, when compared to the susceptible isolates and the reference strain. The work also revealed that not only the *ABC* transporter genes have a huge responsibility in azole drug resistance of *C. glabrata*, but small hydrophobic compounds transport and the lipid metabolism may have responsability [318]. A study of Ferrari and colleagues [128] in transcription profiling with microarrays showed that more than 385 genes are differentially regulated by a selected number of the gain-of-function mutations (GOF) expressed in the same genetic background, with a minimal overlap in co-regulated genes. *CDR1* and *PUP1* were generally upregulated by all tested GOFs (for *PDR1* upregulated and encoding a mitochondrial protein). While both genes mediated azole resistance, their deletions in an azole resistant isolate managed a decline in virulence and a decrease tissue load. Their individual overexpression showed to partially restore phenotypes obtained in clinical isolates [128]. Kaur and colleagues [319] made a screening of a library of 9,216 random insertion mutants and identified a set of 27 genes, which upon mutation, conferred alterations in fluconazole susceptibility in *C. glabrata*. These genes included *ABC* transporters (*PDR5* and *PDR16*), genes involved in retrograde signaling from mitochondria to nucleus (*RTG2*) and genes involved in diverse cellular functions (activation of RNA polymerase II transcription, calcium homeostasis, ribosomal biogenesis and mitochondrial function, nuclear ubiquitin ligase function, and cell wall biosynthesis). Similarly, using a mutant defective in calcium uptake, the same authors noticed that the strains flawed in a putative plasma membrane calcium channel were modestly more susceptible to fluconazole, and revealed a significant loss of viability upon prolonged fluconazole exposure. This result suggests that calcium signaling is necessary for survival of azole stress in *C. glabrata* and that, in the absence of Ca^{2+} signaling, fluconazole has a fungicidal rather than a fungistatic effect on *C. glabrata* [320]. Salazar et al [321] recently sequenced the whole-genome of a fluconazole and voriconazole-resistant clinical isolate (FFUL887) and compared with the genome of the susceptible strain CBS138. The

results revealed important differences in the transcriptional regulator Pdr1. The Pdr1 FFUL887 allele included a K274Q modification not documented in other azole-resistant strains. The transcriptomic profiling demonstrated the upregulation of 92 documented targets of Pdr1 in the FFUL887 strain, which indicated that the K274Q substitution originates a Pdr1 GOF mutant. Additionally, the cells were more susceptible to high concentrations of organic acids at a low pH (4.5) but had no visible effect in tolerance towards other environmental stressors, when the expression of Pdr1 K274Q in the FFUL887 background. Significant differences in the sequence of adhesin-encoding genes were also found, among the two strains, which a substantial adjustment of the expression of genes involved in metabolism of carbohydrates, nitrogen and sulphur in the FFUL887 strain [321].

Another azole-related resistance mechanism is the decreased affinity, or even incapacity, of these drugs to bind to the cells. The high ability to upregulate *ERG11, CDR1* and *PDR1* expression is normally followed by azole exposure [122]. All the genes associated to the biosynthesis of ergosterol are likely to be upregulated in the case of azole pressure, nonetheless, *ERG1, 3, 6, 7, 9* and especially *ERG11* are the most studied. *ERG11*, that converts lanosterol into 4, 4-dimethylcolesta-8,14,24-trienol, is markedly more mentioned as central point on the increase of ergosterol bioproduction, in the response to the azole attack to *C. glabrata* cell membrane [154,216]. Potential mechanisms to the azole resistance include a small affinity of its lanosterol 14-α-demethylase. The resistance mechanisms in *ERG11* occur through the acquisition of point mutations in the gene encoding for the Erg11. Variations have been reported in the *ERG11* gene in *Candida* spp., some of which have been found exclusively in azole-resistant isolates, while others have also been found in susceptible isolates [322–324]. The impact of the modification on the azole resistance depends on the location and the specific substitution and only a few mutations are directly connected to azole resistance. It is known that target gene mutations frequently occur simultaneously and often also conglomerate with other resistance mechanisms [42, 50–52]. Dunkel et al [52] revealed that the GOF mutations in the transcription gene *UPC2* (that regulates *ERG11* expression) lead to extra Erg11 concentration and consequently insufficient azole activity. Also, the overexpression or upregulation of the target enzyme of the azoles is an important resistant mechanism linked to azole drugs [214]. Recently, a study in three different hospitals in Poland determined the mechanisms of resistance to azoles in *C. glabrata* clinical isolates in this country [112]. The authors used a Sensititre Yeast One test and discovered that, from the 81 studied strains, 18 were resistant to fluconazole, and 15 had cross-resistance to all other azoles tested.

RT-qPCR studies showed that 13 of 15 azole-resistant strains presented upregulation of the *CDR1* gene encoding the efflux pump, but no upregulation of expression of the *CDR2*. Also, no upregulation of *ERG11* gene was observed. This study confirms that the gene profile of the resistant isolates of *C. glabrata* azoles is variable among countries and strains, although certain genes are common to be up or downregulated [112]. In another study, Sanglard and colleagues [325] demonstrated that the MDR1 transporter belonging to the MFS is also involved in azole resistance in *Candida* spp. but apparently does not confer resistance to posaconazole, itraconazole, or isavuconazole. Miyazaki et al [320] studied the effects of the calcineurin, a serine-threonine-specific protein phosphatase [326]. This protein emerged as a new target for antifungal therapy, probably because azole antifungals and calcineurin inhibitors have mild synergistic effects against *C. glabrata* wild-type strains [319,327,328]. The results of this group indicated that *C. glabrata* calcineurin mutant presented an augmented susceptibility to azoles and cell wall-damaging agents and had lower virulence. Though the mutant lacking Crz1 presented a cell wall-associated phenotype intermediate to that of the calcineurin mutant and was modestly reduced in virulence, it did not improve azole susceptibility, proposing that calcineurin regulates both Crz1-dependent and independent pathways depending on the type of stress [320]. Chen et al [129] disclosed that *AP1* (which encodes a transcription factor related to stress responses) plays a critical role in reaction to various stresses in *C. glabrata* and decreases the stress through transcriptional activation of its target genes including *FLR1*. The deletion of this gene only caused an amplified sensitivity to fluconazole. *Candida glabrata* clinical isolates are known to have the aldo-keto-reductase superfamily (*AKR*) upregulated in the resistant isolates. RT-qPCR analysis revealed a *AKR* mRNA expression twice from that seen in the sensitive isolates, associated with increased fluconazole and itraconazole resistance, thus suggesting that upregulation of the *AKR* gene might give a new insight into the mechanism of azole resistance[85,130]. Although the isolation of such *C. glabrata* mutants from patients has been rarely reported, Ferrari et al [317] have accomplished to characterize two sequential and related *C. glabrata* isolates recovered from the same patient undergoing azole therapy: BPY40 (azole susceptible) and BPY41 (azole resistant). BPY41 had a mitochondrial dysfunction with upregulation of the *ABC* transporter genes in *C. glabrata*. Testing the virulence of the "petite mutants" in mice with systemic and vaginal murine infection models, the authors showed that, even with *in vitro* growth deficiency, BPY41 was more virulent than BPY40. The authors also found enrichment of oxido-reductive metabolism and the stress response in BPY41, consistent with mitochondrial dysfunction and that certain genes involved in cell wall

adaptation were upregulated in BPY41 compared to BPY40 [317]. Finally, Taff et al [144] identified two glucan transferases and one exo-glucanase that deliver glucan from the cell to the extracellular matrix, playing a biofilm-specific role, by mediating the distribution and organization of mature biofilm matrix, that could be related to drug resistance.

I.3.2 Resistance to polyenes

The first cases of resistance to amphotericin B arose in parallel with the increase in the number of invasive infections caused by several genus of fungi, many of them with primary or intrinsic resistance to amphotericin B and usually associated with a high mortality [329–331]. Although *C. glabrata* is frequently considered to be susceptible to amphotericin B, it has a tendency to have higher minimum inhibitory concentrations (MIC) values to polyenes than *C. albicans* [305,332]. Isolated cases of acquired resistance to amphotericin B have been reported, resulting in infections, particularly in the oncology field, with significantly higher MIC values to amphotericin B, than colonising isolates from immunocompetent patients [333,334]. Polyene resistance is still poorly understood and documented, mostly in *C. glabrata*, but the molecular mechanisms primarily include the replacement of some or all of the polyene-binding sterols, the reorientation or the camouflaging of existing ergosterol and the decrease in the total ergosterol content of the cell [335,336]. Even if resistance to polyenes have not been a main clinical problem so far, *Candida*-spp.-polyene-resistance continues to be described and associated with mutations in *ERG3*, which is directly linked to qualitative and quantitative alterations of membrane lipids and to the lack of ergosterol [324,337,338]. Studying a clinical isolate of *C. glabrata*, Vandeputte and colleagues [152], revealed lower ergosterol content in its membrane comparing the wild type, also finding a nonsense mutation in the *ERG6* gene, leading to a decrease in ergosterol content. Discrepancies of the cell wall were also observed, associated by a developed susceptibility to cell wall-perturbing agents, with a high rate of cell mortality [152]. In other clinical isolate of *C. glabrata* recovered from a patient treated with amphotericin B and with a poor susceptibility to polyenes, it was detected a deficiency of ergosterol and an accumulation of late sterol intermediates, emphasizing a defect in the final steps of the ergosterol pathway. Sequencing exposed a unique missense mutation in *ERG6* (substitution of a cysteine by a phenylalanine in the corresponding protein). RT-qPCR demonstrated an overexpression of the genes that encode enzymes involved in late steps of the ergosterol pathway. The complementation of this strain with a wild-type copy of the *ERG6* gene regenerated the susceptibility to polyenes and the standard morphology [173]. Furthermore, combined

mutations in *ERG11* and in *ERG3* or *ERG5* and single mutations in *ERG2* have also been associated with depletion of ergosterol and amphotericin B resistance in *C. glabrata* [339–342].

I.3.3 Resistance to echinocandins

Echinocandins are presently the first-line antifungal agents in the treatment of candidaemia [302] and, excepting *C. glabrata*, most *Candida* spp. have a low rate (<3%) of echinocandin resistance [139,343,344]. As reflected, *C. glabrata* isolates are recurrently related with decreased susceptibility to other antifungals, particularly fluconazole and other azoles. Over a 10 year-period, echinocandin resistance ascended from 2%–3% to >13% in 2009–2010 in the USA [34]. Likewise, in other US USA hospitals and in a Centers for Disease Control and Prevention (CDC) survey rates of 3.1%–3.6% from 4 USA cities in varying geographic regions were described [139].

In fact, in these reports, echinocandin resistance was also related with multidrug-resistance to azoles in 36% of the echinocandin-resistant strains. Additionally, "hot spot" *FKS* mutations associated with resistance were frequent in those isolates with elevated MICs and more likely to be associated with clinical failure [344]. It is recognized that three mechanisms reduce the echinocandin susceptibility [214,345]: acquired *FKS* mutations [140] which confer low β-1,3-D-glucan synthase sensitivity, higher MIC values, and clinical failure [346]; adaptive stress responses, which result in high cell wall chitin content with a paradoxical growth *in vitro* [347]; and, finally, intrinsic *FKS* mutations, which also result in elevated MIC levels, but in a lower level of reduced β-1,3-D-glucan synthase sensitivity, when compared to the acquired *FKS* mutations [134,346,348]. The *GAS* gene family is also a regulator in production of β-1,3-glucan in this species [90]. *GAS1*, *GAS2* and *GAS5* are a GPI-anchored cell surface proteins [349], involved in production of β-1,3-glucan in *C. glabrata* [142]. A study performed in patients with *C. glabrata* bloodstream infections showed that the resistance to echinocandins increased from 4.9% to 12.3% between 2001 and 2010. In addition, among the 78 fluconazole resistant isolates, 14.1% were resistant to 1 or more echinocandins and almost 8% of the isolates had a *FKS* mutation (*FKS1/FKS2* mutations), which appeared due to a prior echinocandin therapy. Besides, nearly all revealed intermediate or resistant MICs to one echinocandin [156]. In 2008, Thompson III et al [126] performed sequentiation of hot spots, known to confer echinocandin resistance, and the fallouts revealed an F659V substitution within the *FKS2* region of the glucan synthase complex [126]. Curiously, micafungin MICs of *C. glabrata FKS* hot spot mutant isolates were observed to be less elevated than those obtained for the other echinocandins, showing that the efficacy of micafungin could be differentially dependent

on specific *FKS* genes mutations [155]. In another study, a *C. glabrata* gastrointestinal (GI) colonization model was used to explore whether colonized yeast exposed to caspofungin developed resistance mutations causing systemic infection, upon immunosuppression [350]. The authors were able to recover *FKS* mutants from organs, characterized by a novel Fks1-L630R substitution. They concluded that *C. glabrata* echinocandin resistance can arise within the GI tract and that resistant mutants can readily disseminate upon immunosuppression [350]. Shields et al [140] analyzed several echinocandin MICs and found that the average MIC values of caspofungin and anidulafungin were higher for patients who failed therapy. Some *Candida* spp. isolates observed *in vitro* reflect a curious high-dose paradox which is being related to a complex network of pathways, causing slightly elevated MICs, in which cells appear to regain susceptibility at high levels of a drug [351,352]. This has the potential to contribute to clinical resistance [214] and it is extremely important to differentiate these low-level drug tolerance and adaptive mechanisms from the Fks1-mediated mechanisms that have been observed in clinical isolates and can result in treatment failure [214,353]. In a recent report, Zhao et al [354], reported a limited penetration of echinocandin drugs at the site of *Candida* spp. infections, that has also serious implications for clinical outcomes and emergence of resistance in patients with intra-abdominal candidiasis. Arendrup et al [137] described that the echinocandin resistance has been strongly linked to one molecular mechanism in the case of *C. glabrata* in *FKS2* gene (wild-type AA sequences hot spot 1: FLILSLRDP and hot spot 2: DWIRRYTL). It is recognized that, in most of cases, only a single mutation is responsible for this resistance in *Candida* spp., but, although rarely, cases of several alterations are also reported [355]. The level of resistance rests on the specific codon involved, the specific alteration, and in which species it is occurring [356]. Variations in the *in vivo* activity have been detected when comparing the micafungin, caspofungin and anidulafungin against *C. glabrata*, exposing exceptions from the general rule that *FKS1* and *FKS2* hot spot alterations affect all 3 echinocandins [137]. It was reported that only micafungin retained its activity against *C. glabrata* harbouring the Fks2p-S663F alteration in an animal model and that anidulafungin continued clinically effective, while caspofungin failed in a therapy involving a *C. albicans* isolate with heterologous Fks1p- R647R/G and P649P/L double mutations [137,357].

I.3.4 Other antifungal agents' resistance

I.3.4.1 Antimetabolites

This class of antifungals include the 5-fluorocytosine (or flucytosine), and act as an inhibitor of both DNA and RNA synthesis via the intracytoplasmic conversion of 5-fluorocytosine to 5-fluorouracil [358]. Flucytosine is actively transported into the fungal cell by a permease, which is encoded by *FCY2*. The conversion to 5-fluorouracil or to 5-fluorouridine monophosphate by the enzymes cytosine deaminase or uracil phosphoribosyltransferase is encoded by the *FCY1* and *FUR1* genes, respectively, and act by inhibiting transcription, DNA replication, and protein synthesis. The resistance mechanisms emerge quickly if this drug is used in monotherapy, being already described mutations in the *FCY2, FCY1*, and *FUR1* genes [359–361]. Additionally, arginine homeostasis, cell wall remodeling, and the aquaglycer- oporins of the Fps family have been recently reported as new biological processes that affect flucytosine resistance in *C. glabrata* [362].

I.3.4.2 Allilamines and Morpholines

Allylamines (e.g. naftifine, terbinafine) inhibit ergosterol biosynthesis at the level of squalene epoxidase. Morpholines (amorolfine) inhibit the same pathway at a later step [363].

A study developed by Vermitsky and colleagues [108] showed that in a treatment with terbinafine, *ERG11* was upregulated as previously reported but, unlike in azoles case, it had minimal effect on *CDR1* and *PDH1*. A 2009 report [364] revealed that, in *C. albicans*, the resistance to flucytosine and terbinafine is associated to MAT locus homozygosity and multilocus sequence typing clade 1. It was concluded that a common regulatory mechanism may operate to generate resistance to the two classes of agent that inhibit ergosterol biosynthesis, terbinafine and the azoles, but that flucytosine resistance, though still generally associated with MAT homozygosity, is regulated in a different way.

I.4. Cross-resistance, multidrug resistance and extreme multidrug resistance

Furthermost multidrug resistance (MDR) on *Candida* spp. infections are associated to isolates belonging to species with intrinsic resistance. MDR species that have no intrinsic resistance is rare, as, generally, it requires the gaining of several resistance mechanisms and these frequently come at ability costs [365,366]. *C. glabrata* has emerged as a major health threat since it also rapidly acquires resistance to multiple drug classes and to drugs within the same antifungal drug class (cross-resistance). The use of echinocandins and increase of cross-resistance is originating

apprehension, for the signs of MDR amongst azoles and echinocandins have been reported [164,356,367,368]. Clinical isolates obtained from patients in several epidemiological studies show that not only MDR has been described in isolated events, but also cross-resistance among more than one class of antifungal drugs is growing [369]. As a result, 11.1% to 58.3% of *C. glabrata* isolates resistant to echinocandins showed to also have resistance to fluconazole or other azoles [164,367,370]. Also, *C. glabrata* has been pointedly related to a prior fluconazole exposure and, when causing invasive candidiasis, to a rapid echinocandin therapy without de-escalation [371–374]. Although this demonstrates that, in a prolonged and more broad-spectrum antifungal exposure, *C. glabrata* is particularly disposed to develop MDR, other mechanisms may contribute to the overrepresentation of this species among MDR *Candida* spp.. Very recently, Healey and colleagues [375] demonstrated a mutator phenotype caused by a mismatch repair defect which is prevalent in *C. glabrata* clinical isolates. These strains possess alterations in mismatch repair gene *MSH2* which lead them to display an advanced predisposition to develop resistance to fluconazole, echinocandin, and amphotericin B treatment *in vitro* and in a mouse model of colonization. Also, the authors have found that 55% of all *C. glabrata*, which are recovered from patients, have these genetic characteristics, which is clinically very concerning. The genetic mechanism involved in this process supports the acquisition of resistance to multiple antifungals, partially explaining the higher rates of triazole and MDR associated with *C. glabrata* [375]. Likewise, *MSH2* mutations were found among clinical isolates in France [376]. The outcomes of these studies may help to clarify why echinocandin resistance in *C. glabrata* in some studies has been concomitant to prior fluconazole exposure, even if the drug targets and resistance mechanisms are entirely different [377]. The repercussions of these results are worrying, since it raises the question if fluconazole prescription in the primary healthcare sector, for it is a probable source of MDR in *C. glabrata*. In two studies, a high degree of echinocandin-resistant *C. glabrata* in the oral microflora after candidemia treatment with ≥7 days of echinocandins, and among *C. glabrata* isolates from abdominal candidiasis were reported [378,379]. These results indicate two phenomena: the subtherapeutic drug concentrations (that are possibly associated to the high protein binding of echinocandin drugs) at mucosal surfaces and in focal infections might enable the resistance development; the *Candida* spp. biofilm of the oral cavity or on the intraabdominal devices may be involved in resistance selection, through drug sequestration and lower drug exposure [276].

MDR of *C. glabrata* of echinocandins and amphotericin B has also been described [380]. The estimate on the percentage of non-susceptible or resistant *C. glabrata* clinical isolates against four

antifungal drugs used in clinical practice, across several geographic regions are: fluconazole, 3.4-70; amphotericin B, 2.5-60; caspofungin, 1.3-16.2 and 5-flucytosine, 0.8-35, which are evidently concerning numbers [145,369,380–399]. Additionally, the latest MDR between amphotericin B and azoles or caspofungin in *Candida* spp. are increasingly worrying [324,339,400–402]. Not in all cases, but in most of them, this MDR depend on the activity of *ABC* transporters and the *MFS* [403], which are known to be regulated by the Pdr1 transcription factor, identified as the major regulator of multidrug resistance in *C. glabrata* [108]. As previously explained, *C. glabrata* expresses three *ABC* transporters (*CDR1*, *CDR2* and *SNQ2*). Studies revealed that the deletion of *CDR1* in an azole-resistant strain, leads to the intracellular accumulation of fluconazole and hypersusceptibility to other azoles. The additional *CDR2* deletion worsens this phenotype [128,404]. Finally, the deletion of *SNQ2* leads to an amplified susceptibility to several azoles but also to 4-nitroquinoline-*N*-oxide, in an azole-resistant strain [405]. Nishikawa and colleagues [106] have identified an activator-targeted KIX domain in the human MED15 Mediator subunit that is structurally conserved in Gal11/Med15 Mediator subunits in fungi. This Gal11/Med15 KIX domain is involved in Pdr1 orthologues and in the clinically important in *C. glabrata* MDR pathogenesis [111]. The authors implemented a sequential biochemical and *in vivo* high-throughput screens to identify small-molecule inhibitors of the interaction of the *C. glabrata* Pdr1 activation domain with the *C. glabrata* Gal11A KIX domain, which is linked to the *C. glabrata* multidrug resistance. Results have shown that iKIX1 inhibits Pdr1-dependent gene activation and re-sensitizes drug-resistant *C. glabrata* to azole antifungals *in vitro* and in animal models for disseminated and urinary tract *C. glabrata* infection [106]. The sirtuins Sir2 and Hst1 control the expression of several genes including adhesins required for host colonization and niacin transporters needed for growth in *C. glabrata*. Having the knowledge that these sirtuins can be inactivated during infection, Orta-Zavalza et al [406] verified if their inhibition could change the response of *C. glabrata* to other stressful conditions. The results showed that a deletion of *HST1* reduced the susceptibility of *C. glabrata* to fluconazole and hydrogen peroxide. Pdr1 and *CDR1* mediated the fluconazole resistance phenotype of the *hst1*Δ cells, while the transcriptional activator Msn4 and the catalase Cta1 were required to provide oxidative stress resistance. Also, the authors stated that the transcription factor Sum1 interacts with Hst1 and participate in the regulation of these genes. The findings validate that *Hst1* acts as a regulator of stress resistance associated-genes [406].

Candida spp. seem to tolerate the stress induced by weak acids, which appear to be a key factor in their persistence and virulence in antifungal drugs. *MFS* transporters are integrated in to two

families: the DHA1 (drug: H^+ antiporter family 1), with 12 transmembrane domains, and the DHA2 (drug: H^+ antiporter family 2), with 14 transmembrane domains. Other transporters, Qdr2 and Tpo3, have also been studied [403]. The first was acknowledged as a cause of resistance to imidazoles (e.g. clotrimazole, miconazole, tioconazole and ketoconazole) and proved to play an active part in the efflux of these drugs. Its expression was found to be stimulated in clotrimazole-stressed cells, under Pdr1 control [407]. The second demonstrated to be linked to the resistance to both imidazoles and triazoles (e.g. fluconazole), and to the polyamine spermine, found in high concentrations in the urogenital tract. The authors also found that *TPO3* was upregulated in *C. glabrata* cells exposed to spermine, in a Pdr1-dependent manner [403]. Likewise, Tpo3 appears to be associated to the efflux of azoles and spermine, and the control of the intracellular concentration of this polyamine appears to be important for azole resistance [408]. Another *MFS* H^+ antiporter, Aqr1, from *C. glabrata,* was also identified by Costa et al [409]. This *MFS* antiporter is a determinant of resistance to acetic acid, flucytosine and clotrimazole (frequently found in the vaginal mucosa and probably contributing to the persistence in this niche). It is known that these antifungals act synergistically with acetic acid against this pathogen. Aqr1 (located in plasma membrane and in the membrane vesicles) was suggested to play a role in intermediating the extrusion of chemical compounds, dropping the intracellular accumulation of 3H-flucytosine and, in a minor degree, of 3H-clotrimazole, which is reliable with a direct role in antifungal drug efflux. When an *AQR1* deletion was performed, no effect could be noticed on the intracellular accumulation of 14C-acetic acid, proposing that its role in acetic acid resistance may be indirect, possibly through the transport of a so far undisclosed physiological substrate. The pre-exposure to flucytosine or clotrimazole was found to make *C. glabrata* cells more tolerant to acetic acid stress. Therefore, Costa et al [409] showed that Aqr1 is an antifungal drug resistance determinant and it may play an essential part in *C. glabrata* persistent colonization and MDR.

Hull et al [410] identified a clinical isolate of *C. glabrata* (CG156) that displayed flocculent growth and cross-resistance to fluconazole, voriconazole, and amphotericin B. In this work, CG156 was found to be a low-efflux isolate and when grown on sterol-supplemented, its cultures reached higher cell densities, with shorter lag phases, showing variations in cellular sterol composition that did not affect its azole-resistant phenotype. When this isolate was grown in the presence of ergosterol, it demonstrated increased sensitivity to the polyene and when grown with cholesterol it became more resistant. The results, thus, indicate that some clinical isolates might persist as slow-growing agents of chronic infections, possibly since they can survive without sterol auxotrophy; possess mutated

Erg11; lack cellular ergosterol (high-level resistance to polyenes); can opportunistically exploit a wide spectrum of host/environmental sterols for growth. The authors also indicate that the altered cellular sterol composition of CG156 may affect intracellular signalling and trafficking pathways, as the efflux machinery [404,411,412] and any transport proteins that are proposed to mediate azole import via facilitated diffusion [413]. Other cases of induced MDR to azole drugs in *C. glabrata* were related to resistance against both azoles and amphotericin B [401,402,410] and related to prochloraz (an agricultural antifungal). The original mechanism responsible for this phenomenon was found to be the upregulation of multidrug transporters [414]. Also in *C. glabrata*, Flr1 has proved to be involved in the resistance of benomyl (a pesticide used in agriculture), but no connection was found between this transporter and antifungal resistance [129].

Finally, extreme MDR has been reported in a rare number of patients undergoing long-term and alternating antifungal therapy. It was observed in a *C. albicans* clinical isolate with azole, echinocandin, and amphotericin B resistance from a patient with mucosal infection over a 5-year period [49], and azole, flucytosine, and echinocandin resistance was acquired in *C. glabrata* due to the acquisition of mutations in *FUR1* and *FKS2* and overexpression of *CDR1* and *CDR2* throughout 20 weeks of antifungal therapy in a hematopoietic stem cell transplant recipient [66].

I.5. Alternatives approaches for the treatment of infections related to *Candida glabrata*

As it was stated, the presence of biofilms reduces the possibility of the elimination of microorganisms by the host defense mechanism and by antifungal drugs. The identification of efficient alternative therapies to the current antifungal agents is, thus, crucial.

Several research groups have confirmed that *Candida* spp. cells present in biofilms have high levels of resistance to the most commonly used antiseptics or antifungal agents [415–417]. Recent approaches are being followed and evaluated, comprising the photodynamic therapy [418–423], natural methodologies as the exploitation of the antifungal properties of plant essential oils and extracts [424–430] and honey [431–439], the use of probiotics [417,440–460] and prebiotics [461–464], and the development of novel compounds as antifungal drugs or immunotherapies [465–482] or the search for possible new drug targets [483–489].

References

1. Atlas, R.; Bartha, R. *Microbial Ecology*; Benjamin; Cummings, Eds.; 4th ed.; Science Publishing: Menlo Park, CA, 1997.
2. Costerton, J. W.; Lewandowski, Z.; Caldwell, D. E.; Korber, D. R.; Lappin-Scott, H. M. Microbial Biofilms. *Annu. Rev. Microbiol.* **1995**, *49*, 711–745.
3. Potera, C. Studying slime. *Environ. Health Perspect.* **1998**, *106*, A604-6.
4. Jass, J.; Surman, S.; Walker, S. *Medical Biofilms Detection, Prevention, and Control. Chichester*; John Wiley & Sons, Ltd: UK, 2003.
5. Gulati, M.; Nobile, C. J. Candida albicans biofilms: Development, regulation, and molecular mechanisms. *Microbes Infect.* **2015**, *18*, 310–321.
6. Leeuwenhoek A., van Microscopical observations about animals in the scurf of the teeth. *Philos Trans R Soc L. B Biol Sci* **1684**, *14:*
7. Henrici, A. T. Studies of Freshwater Bacteria: I. A Direct Microscopic Technique. *J. Bacteriol.* **1933**, *25*, 277–87.
8. Rogovska T; Lazareva, M. Microbiologicalcharacteristics of biofilm purifying hydrogensulphide containing sewage. *Mikrobiologya* **1961**.
9. Rogovska, T.; Lazareva, M. Microbiological characteristics of biofilm purifying hydrogen sulphide containing sewage. **1961**.
10. Høiby, N. Pseudomonas aeruginosa infection in cystic fibrosis., University of Copenhagen, 1977.
11. McCoy, W. F.; Bryers, J. D.; Robbins, J.; Costerton, J. W. Observations of fouling biofilm formation. *Can. J. Microbiol.* **1981**, *27*, 910–7.
12. Costerton, J. W.; Cheng, K. J.; Geesey, G. G.; Ladd, T. I.; Nickel, J. C.; Dasgupta, M.; Marrie, T. J. Bacterial Biofilms in Nature and Disease. *Annu. Rev. Microbiol.* **1987**, *41*, 435–464.
13. Costerton, J. W.; Lewandowski, Z.; DeBeer, D.; Caldwell, D.; Korber, D.; James, G. Biofilms, the customized microniche. *J. Bacteriol.* **1994**, *176*, 2137–42.
14. Costerton, J. W.; Stewart, P. S.; Greenberg, E. P. Bacterial biofilms: a common cause of persistent infections. *Science* **1999**, *284*, 1318–22.
15. Bjarnsholt, T. The role of bacterial biofilms in chronic infections, 2013, Vol. 121.
16. Costerton JW. Replacement of acute planctonic by chronic biofilm diseases. In *The Biofilm Primer.*; Eckey, C., Ed.; Springer US: New York, 2007; p. 129–61.
17. Costerton, J. W.; Geesey, G. G.; Cheng, K. J. How bacteria stick. *Sci. Am.* **1978**, *238*, 86–95.
18. Bayles, K. W. The biological role of death and lysis in biofilm development. *Nat. Rev. Microbiol.* **2007**, *5*, 721–726.
19. Fidel, P.; Vazquez, J.; Sobel, J. Candida glabrata: review of epidemiology, pathogenesis, and clinical disease with comparison to C. albicans. *Clin. Microbiol. Rev.* **1999**, *12*, 80–96.
20. Sasani, E.; Khodavaisy, S.; Agha Kuchak Afshari, S.; Darabian, S.; Aala, F.; Rezaie, S. Pseudohyphae formation in Candida glabrata due to CO2 exposure. *Curr. Med. Mycol.* **2017**, *2*, 49–52.
21. Csank, C.; Haynes, K. Candida glabrata displays pseudohyphal growth. *FEMS Microbiol. Lett.* **2000**, *189*, 115–20.
22. Brunke, S.; Seider, K.; Fischer, D.; Jacobsen, I. D.; Kasper, L.; Jablonowski, N.; Wartenberg, A.; Bader, O.; Enache-Angoulvant, A.; Schaller, M.; D 'enfert, C.; Hube, B.; Brunke, S.; Seider, K.; Fischer, D.; Id, J.; Kasper, L. One Small Step for a Yeast -Microevolution within Macrophages Renders Candida glabrata Hypervirulent Due to a Single Point Mutation. *PLoS Pathog.* **2014**, *10*, e1004478.
23. Dujon, B.; Sherman, D.; Fischer, G.; Durrens, P.; Casaregola, S.; Lafontaine, I.; de Montigny, J.; Marck, C.; Neuvéglise, C.; Talla, E.; Goffard, N.; Frangeul, L.; Aigle, M.; Anthouard, V.; Babour, A.; Barbe, V.; Barnay, S.; Blanchin, S.; Beckerich, J.-M.; Beyne, E.; Bleykasten, C.; Boisramé, A.; Boyer, J.; Cattolico, L.; Confanioleri, F.; de Daruvar, A.; Despons, L.; Fabre, E.; Fairhead, C.; Ferry-Dumazet, H.; Groppi, A.; Hantraye, F.; Hennequin, C.; Jauniaux, N.; Joyet, P.; Kachouri, R.; Kerrest, A.; Koszul, R.; Lemaire, M.; Lesur, I.; Ma, L.; Muller, H.; Nicaud, J.-M.; Nikolski, M.; Oztas, S.; Ozier-Kalogeropoulos, O.; Pellenz, S.; Potier, S.; Richard, G.-F.; Straub, M.-L.; Suleau, A.; Swennen, D.; Tekaia, F.; Wésolowski-Louvel, M.; Westhof, E.; Wirth, B.; Zeniou-Meyer, M.; Zivanovic, I.; Bolotin-Fukuhara, M.; Thierry, A.; Bouchier, C.; Caudron, B.; Scarpelli, C.; Gaillardin, C.; Weissenbach, J.; Wincker, P.; Souciet, J.-L. Genome evolution in yeasts. *Nature* **2004**, *430*, 35–44.
24. Wolfe, K. H.; Shields, D. C. Molecular evidence for an ancient duplication of the entire yeast genome. *Nature* **1997**, *387*, 708–713.
25. Cen, Y.; Timmermans, B.; Souffriau, B.; Thevelein, J. M.; Van Dijck, P. Comparison of genome engineering using the CRISPR-Cas9 system in C. glabrata wild-type and lig4 strains. *Fungal Genet. Biol.* **2017**, *107*, 44–50.
26. Butler, G.; Rasmussen, M. D.; Lin, M. F.; Santos, M. A. S.; Sakthikumar, S.; Munro, C. A.; Rheinbay, E.; Grabherr, M.; Forche, A.; Reedy, J. L.; Agrafioti, I.; Arnaud, M. B.; Bates, S.; Brown, A. J. P.; Brunke, S.; Costanzo, M. C.; Fitzpatrick, D. A.; de Groot, P. W. J.; Harris, D.; Hoyer, L. L.; Hube, B.; Klis, F. M.; Kodira, C.; Lennard, N.; Logue, M.

E.; Martin, R.; Neiman, A. M.; Nikolaou, E.; Quail, M. A.; Quinn, J.; Santos, M. C.; Schmitzberger, F. F.; Sherlock, G.; Shah, P.; Silverstein, K. A. T.; Skrzypek, M. S.; Soll, D.; Staggs, R.; Stansfield, I.; Stumpf, M. P. H.; Sudbery, P. E.; Srikantha, T.; Zeng, Q.; Berman, J.; Berriman, M.; Heitman, J.; Gow, N. A. R.; Lorenz, M. C.; Birren, B. W.; Kellis, M.; Cuomo, C. A. Evolution of pathogenicity and sexual reproduction in eight Candida genomes. *Nature* **2009**, *459*, 657–662.

27. Brunke, S.; Hube, B. Two unlike cousins: Candida albicans and C. glabrata infection strategies. *Cell. Microbiol.* **2013**, *15*, 701–8.

28. Introduction and historical perspectives. In *Candida and Candidiasis*; Calderone, R., Ed.; ASM Press, Washington D.C., 2002; pp. 15–25.

29. Larone, H. *Medically important fungi: a guide to identification.*; 4th ed.; Washington: ASM Press, 2002.

30. Silva, S.; Negri, M.; Henriques, M.; Oliveira, R.; Williams, D. W.; Azeredo, J. Candida glabrata, Candida parapsilosis and Candida tropicalis: biology, epidemiology, pathogenicity and antifungal resistance. *FEMS Microbiol. Rev.* **2012**, *36*, 288–305.

31. Odds, F. *Candida and Candidiasis*; 2nd ed.; London: Baillierir Tindall., 1988.

32. Butler, G.; Kenny, C.; Fagan, A.; Kurischko, C.; Gaillardin, C.; Wolfe, K. H. Evolution of the MAT locus and its Ho endonuclease in yeast species. *Proc. Natl. Acad. Sci.* **2004**, *101*, 1632–1637.

33. Kaur, R.; Domergue, R.; Zupancic, M. L.; Cormack, B. P. A yeast by any other name: Candida glabrata and its interaction with the host. *Curr. Opin. Microbiol.* **2005**, *8*, 378–384.

34. Colombo, A. L.; Guimaraes, T.; Silva, L. R. B. F.; Monfardini, L. P. de A.; Cunha, A. K. B.; Rady, P.; Alves, T.; Rosas, R. C. Prospective Observational Study of Candidemia in Sao Paulo, Brazil: Incidence Rate, Epidemiology, and Predictors of Mortality •. *Infect. Control Hosp. Epidemiol.* **2007**, *28*, 570–576.

35. Hittinger, C. T.; Rokas, A.; Carroll, S. B. Parallel inactivation of multiple GAL pathway genes and ecological diversification in yeasts. *Proc. Natl. Acad. Sci.* **2004**, *101*, 14144–14149.

36. Kaur, R.; Domergue, R.; Zupancic, M. L.; Cormack, B. P. A yeast by any other name: Candida glabrata and its interaction with the host. *Curr. Opin. Microbiol.* **2005**, *8*, 378–384.

37. Gerwien, F.; Safyan, A.; Wisgott, S.; Brunke, S.; Kasper, L.; Hube, B. The fungal pathogen Candida glabrata does not depend on surface ferric reductases for iron acquisition. *Front. Microbiol.* **2017**, *8*.

38. Sharma, V.; Purushotham, R.; Kaur, R. The Phosphoinositide 3-Kinase Regulates Retrograde Trafficking of the Iron Permease CgFtr1 and Iron Homeostasis in Candida glabrata. *J. Biol. Chem.* **2016**, *291*, 24715–24734.

39. Bongomin, F.; Gago, S.; Oladele, R.; Denning, D. Global and Multi-National Prevalence of Fungal Diseases—Estimate Precision. *J. Fungi* **2017**, *3*, 57.

40. Cole, D. C.; Govender, N. P.; Chakrabarti, A.; Sacarlal, J.; Denning, D. W. Series Fungal infections 8 Improvement of fungal disease identification and management: combined health systems and public health approaches. *Lancet Infect. Dis.* **2017**.

41. GAFFI. Burden of disease maps. Global Action Fund for Fungal Infections. http://www.gaffi.org/why/burden-of-disease-maps (accessed Feb 5, 2018).

42. Farmakiotis, D.; Kontoyiannis, D. P. Epidemiology of antifungal resistance in human pathogenic yeasts: current viewpoint and practical recommendations for management. *Int. J. Antimicrob. Agents* **2017**, *50*.

43. Colombo, A. L.; Júnior, J. N. D. A.; Guinea, J. Emerging multidrug-resistant Candida species. *Curr. Opin. Infect. Dis.* 2017, *30*.

44. Rodrigues, C. F.; Silva, S.; Henriques, M. Candida glabrata: A review of its features and resistance. *Eur. J. Clin. Microbiol. Infect. Dis.* **2014**, *33*, 673–688.

45. Bassetti, M.; Righi, E.; Costa, A.; Fasce, R.; Molinari, M. P.; Rosso, R.; Pallavicini, F. B.; Viscoli, C. Epidemiological trends in nosocomial candidemia in intensive care. *BMC Infect. Dis.* **2006**, *6*, 21.

46. Hasan, F.; Xess, I.; Wang, X.; Jain, N.; Fries, B. C. Biofilm formation in clinical Candida isolates and its association with virulence. *Microbes Infect.* **2009**, *11*, 753–61.

47. Trouvé, C.; Blot, S.; Hayette, M. P.; Jonckheere, S.; Patteet, S.; Rodriguez-Villalobos, H.; Symoens, F.; Van Wijngaerden, E.; Lagrou, K. Epidemiology and reporting of candidaemia in Belgium: a multi-centre study. *Eur. J. Clin. Microbiol. Infect. Dis.* **2017**, *36*.

48. Chapman, B.; Slavin, M.; Marriott, D.; Halliday, C.; Kidd, S. E.; Arthur, I.; Bak, N.; Heath, C. H.; Kennedy, K.; Morrissey, C. O.; Sorrell, T. C.; van Hal, S.; Keighley, C.; Goeman, E.; Underwood, N.; Hajkowicz, K.; Hofmeyr, A.; Leung, M.; Macesic, N.; Botes, J.; Blyth, C.; Cooley, L.; George, C. R.; Kalukottege, P.; Kesson, A.; McMullan, B.; Baird, R.; Robson, J.; Korman, T. M.; Pendle, S.; Weeks, K.; Liu, E.; Cheong, E.; Chen, S. C. A.; Daveson, K.; Kennedy, K.; Wilson, H.; Underwood, N.; Hajkowicz, K.; Thomas, M.; Robson, J.; Peachey, G.; Cheong, E.; Gottlieb, T.; Lui, E.; Pendle, S.; Kalukottege, R. P.; Hofmeyr, A.; Jozwiak, F.; George, C. R.; Bell, S. M.; van Hal, S.; Menon, V.; Kotsiou, G.; Weeks, K.; George, C. R.; Bell, S. M.; Marriott, D.; Goeman, E.; McMullan, B.; Kesson, A.; Botes, J.; Chen, S. C. A.; Sorrell, T. C.; Keighley, C.; Halliday, C.; Baird, R.; Marshall, C.; Bak, N.; Ferguson, C.; Kidd, S. E.; Cooley, L.; Morrissey, C. O.; Korman, T. M.; Bryant, M.; Slavin, M.; Reed, C.; Slavin, M.; Darvall, J.; Maddigan, V.; Blyth, C.; Leung, M.;

Arthur, I.; Heath, C. H.; Boan, P. Changing epidemiology of candidaemia in Australia. *J. Antimicrob. Chemother.* **2017**, *72*, 1103–1108.
49. Bassetti, M.; Garnacho-Montero, J.; Calandra, T.; Kullberg, B.; Dimopoulos, G.; Azoulay, E.; Chakrabarti, A.; Kett, D.; Leon, C.; Ostrosky-Zeichner, L.; Sanguinetti, M.; Timsit, J. F.; Richardson, M. D.; Shorr, A.; Cornely, O. A. Intensive care medicine research agenda on invasive fungal infection in critically ill patients. *Intensive Care Med.* 2017.
50. Rodriguez, L.; Bustamante, B.; Huaroto, L.; Agurto, C.; Illescas, R.; Ramirez, R.; Diaz, A.; Hidalgo, J. A multi-centric study of Candida bloodstream infection in Lima-Callao, Peru: Species distribution, antifungal resistance and clinical outcomes. *PLoS One* **2017**, *12*.
51. Krcmery, V.; Barnes, A. J. Non-albicans Candida spp. causing fungaemia: pathogenicity and antifungal resistance. *J. Hosp. Infect.* **2002**, *50*, 243–260.
52. Krcmery, V. Torulopsis glabrata an emerging yeast pathogen in cancer patients. *Int. J. Antimicrob. Agents* **1999**, *11*, 1–6.
53. Tortorano, A. M.; Kibbler, C.; Peman, J.; Bernhardt, H.; Klingspor, L.; Grillot, R. Candidaemia in Europe: epidemiology and resistance. *Int. J. Antimicrob. Agents* **2006**, *27*, 359–66.
54. Sardi, J. C. O.; Scorzoni, L.; Bernardi, T.; Fusco-Almeida, A. M.; Mendes Giannini, M. J. S. Candida species: Current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. *J. Med. Microbiol.* **2013**, *62*, 10–24.
55. Lim, C. S.-Y.; Rosli, R.; Seow, H. F.; Chong, P. P. Candida and invasive candidiasis: back to basics. *Eur. J. Clin. Microbiol. Infect. Dis.* **2012**, *31*, 21–31.
56. Abi-Said, D.; Anaissie, E.; Uzun, O.; Pinzcowski, H.; Vartivarian, S. The epidemiology of hematogeneous candidiasis caused by different Candida species. *Clin Infect Dis* **1997**, *24*, 1122–28.
57. Martins, M. M. D.; Lozano-Chiu, M.; Rex, J. H. J. H.; Lozano- Chiu, M.; Rex, J. H. J. H.; Lozano-Chiu, M.; Rex, J. H. J. H. Declining rates of oropharyngeal candidiasis and carriage of Candida albicans associated with trends toward reduced rates of carriage of fluconazole-resistant C. albicans in human immunodeficiency virus-infected patients. *Clin. Infect. Dis.* **1998**, *27*, 1291–1294.
58. Beck-Sague, C.; Jarvis, W. National nosocomial infectious surveillance system. Secular trends in the epidemiology of nosocomial fungal infections in the United States, 1980–1990. *J Infect Dis* **1993**, *167*, 1247–51.
59. Rees, J. R.; Pinner, R. W.; Hajjeh, R. A.; Brandt, M. E.; Reingold, A. L. The epidemiological features of invasive mycotic infections in the San Francisco Bay area, 1992–1993: results of population-based laboratory active surveillance. *Clin Infect Dis* **1998**, *7*, 1138–47.
60. Pfaller, M.; Jones, R.; Messer, S.; Edmond, M.; Wenzel, R. National surveillance of nosocomial blood stream infection due to species of Candida other than Candida albicans: frequency of occurrence and antifungal susceptibility in the SCOPE Program. *Diagn Microbiol Infect Dis* **1998**, *30*, 121–29.
61. Pfaller, M. A.; Jones, R. N.; Doern, G. V; Sader, H. S.; Messer, S. A.; Houston, A.; Coffman, S.; Hollis, R. J. Bloodstream infections due to Candida species: SENTRY antimicrobial surveillance program in North America and Latin America, 1997–1998. *Antimicrob Agents Chemother* **2000**, *44*, 747–51.
62. Gumbo, T.; Isada, C.; Hall, G.; Karafa, M.; Gordon, S. Candida glabrata fungemia. Clinical features of 139 patients. *Med.* **1999**, *78*, 220–27.
63. Pfaller, M.; Jones, R.; Doern, G.; Fluit, A.; Verhoef, J.; Sader, H.; Messer, S.; Houston, A.; Coffman, S.; Hollis, R. International surveillance of blood stream infections due to Candida species in the European SENTRY Program: species distribution and antifungal susceptibility including the investigational triazole and echinocandin agents. SENTRY Participant Group (Euro). *Diagn Microbiol Infect Dis* **1999**, *35*, 19–25.
64. Rodriguez-Tudela, J.; Cuenca-Estrella, M. Estudio multicéntrico sobre funguemias por levaduras en España (abril-junio de 1997). Grupo de trabajo para el estudio de las funguemias. *Rev Clin Esp* **1999**, *199*, 356–61.
65. Pfaller, M.; Diekema, D.; Jones, R.; Sader, H.; Fluit, A.; Hollis, R.; Messer, S.; Group, T. S. P. International surveillance of bloodstream infections due to Candida species: frequency of occurrence and in vitro susceptibilities to fluconazole, ravuconazole, and voriconazole of isolates collected from 1997 through 1999 in the SENTRY antimicrob surv. *J Clin Microbiol* **2001**, *39*, 3254–59.
66. Wingard, J. R. . Importance of Candida species other than C. albicans as pathogens in oncology patients. *Clin. Infect. Dis.* **1995**, *20*, 115–25.
67. Rex, J.; Pfaller, M.; Barry, A.; Nelson, P.; Webb, C. Antifungal susceptibility testing of isolates from a randomized, multicenter trial of fluconazole versus amphotericin B as treatment of nonneutropenic patients with candidemia. *Antimicrob Agents Chemother* **1995**, *39*, 40–44.
68. Abbas, J.; Bodey, G. P.; Hanna, H. A.; Mardani, M.; Girgawy, E.; Abi-Said, D.; Whimbey, E.; Hachem, R.; Raad, I. Candida krusei fungemia. An escalating serious infection in immunocompromised patients. *Arch Intern Med* **2000**, *160*, 2659–64.
69. Vazquez, J. A.; Sanchez, V.; Dmuchowski, C.; Dembry, L. M.; Sobel, J. D.; Zervos, M. J. Nosocomial acquisition of Candida albicans: an epidemiologic study. *J. Infect. Dis.* **1993**, *168*, 195–201.

70. Vazquez, J. A.; Dembry, L. M.; Sanchez, V.; Vazquez, M. A.; Sobel, J. D.; Dmuchowski, C.; Zervos, M. J. Nosocomial Candida glabrata colonization: an epidemiologic study. *J. Clin. Microbiol.* **1998**, *36*, 421–6.
71. Martins, M.; Henriques, M.; Ribeiro, A. P.; Fernandes, R.; Gonçalves, V.; Seabra, Á.; Azeredo, J.; Oliveira, R. Oral Candida carriage of patients attending a dental clinic in Braga, Portugal. *Rev. Iberoam. Micol.* **2010**, *27*, 119–124.
72. Masiá Canuto, M.; Gutiérrez Rodero, F.; Ortiz de la Tabla Ducasse, V.; Hernández Aguado, I.; Martín González, C.; Sánchez Sevillano, A.; Martín Hidalgo, A. Determinants for the Development of Oropharyngeal Colonization or Infection by Fluconazole-Resistant Candida Strains in HIV-Infected Patients. *Eur. J. Clin. Microbiol. Infect. Dis.* **2000**, *19*, 593–601.
73. Redding, S. W.; Kirkpatrick, W. R.; Coco, B. J.; Sadkowski, L.; Fothergill, A. W.; Rinaldi, M. G.; Eng, T. Y.; Patterson, T. F. Candida glabrata oropharyngeal candidiasis in patients receiving radiation treatment for head and neck cancer. *J. Clin. Microbiol.* **2002**, *40*, 1879–81.
74. De Bernardis, F.; Chiani, P.; Ciccozzi, M.; Pellegrini, G.; Ceddia, T.; D'Offizzi, G.; Quinti, I.; Sullivan, P. A.; Cassone, A. Elevated aspartic proteinase secretion and experimental pathogenicity of Candida albicans isolates from oral cavities of subjects infected with human immunodeficiency virus. *Infect. Immun.* **1996**, *64*, 466–71.
75. Fidel, P. L.; Cutright, J. L.; Tait, L.; Sobel, J. D. A murine model of Candida glabrata vaginitis. *J. Infect. Dis.* **1996**, *173*, 425–31.
76. Negri, M.; Silva, S.; Henriques, M.; Oliveira, R. Insights into Candida tropicalis nosocomial infections and virulence factors. *Eur J Clin Microbiol Infect Dis* **2012**, *31*, 1399–1412.
77. Silva, S.; Henriques; Martins, A.; Oliveira, R.; Williams, D.; Azeredo, J. Biofilms of non-Candida albicans Candida species: quantification, structure and matrix composition. *Med Mycol.* **2009**, *47*, 681–689.
78. Fonseca, E.; Silva, S.; Rodrigues, C. F. C. F.; Alves, C. T. C. T.; Azeredo, J.; Henriques, M. Effects of fluconazole on Candida glabrata biofilms and its relationship with ABC transporter gene expression. *Biofouling* **2014**, *30*, 447–57.
79. Rodrigues, C. F.; Silva, S.; Azeredo, J.; Henriques, M. Candida glabrata's recurrent infections: biofilm formation during Amphotericin B treatment. *Lett. Appl. Microbiol.* **2016**, *63*, 77–81.
80. Iraqui, I.; Garcia-Sanchez, S.; Aubert, S.; Dromer, F.; Ghigo, J.-M.; d'Enfert, C.; Janbon, G. The Yak1p kinase controls expression of adhesins and biofilm formation in Candida glabrata in a Sir4p-dependent pathway. *Mol. Microbiol.* **2005**, *55*, 1259–71.
81. Klotz, S. A.; Drutz, D. J.; Zajic, J. E. Factors Governing Adherence of Candida Species to Plastic Surfaces. *Infect. Immun.* **1985**, *50*, 97–101.
82. Hazen, K. C.; Plotkin, B. J.; Klimas, D. M. Influence of growth conditions on cell surface hydrophobicity of Candida albicans and Candida glabrata. *Infect. Immun.* **1986**, *54*, 269–71.
83. Kikutani, H.; Makino, S. The murine autoimmune diabetes model: NOD and related strains. *Adv. Immunol.* **1992**, *51*, 285–322.
84. De Las Peñas, A.; Pan, S.-J.; Castaño, I.; Alder, J.; Cregg, R.; Cormack, B. P. Virulence-related surface glycoproteins in the yeast pathogen Candida glabrata are encoded in subtelomeric clusters and subject to RAP1- and SIR-dependent transcriptional silencing. *Genes Dev.* **2003**, *17*, 2245–58.
85. Castano, I.; Pan, S.; Zupancic, M.; Hennequin, C.; Dujon, B.; Cormack, B. Telomere length control and transcriptional regulation of subtelomeric adhesins in Candida glabrata. *Mol. Microbiol.* **2005**, *55*, 1246–1258.
86. Sundstrom, P. Adhesion in Candida spp. *Cell. Microbiol.* **2002**, *4*, 461–9.
87. Filler, S. G. Candida–host cell receptor–ligand interactions. *Curr. Opin. Microbiol.* **2006**, *9*, 333–339.
88. Jayatilake, J. A. M. S.; Samaranayake, Y. H.; Cheung, L. K.; Samaranayake, L. P. Quantitative evaluation of tissue invasion by wild type, hyphal and SAP mutants of Candida albicans, and non-albicans Candida species in reconstituted human oral epithelium. *J. Oral Pathol. Med.* **2006**, *35*, 484–491.
89. Riera, M.; Mogensen, E.; d'Enfert, C.; Janbon, G.; d'Enfert, C.; Janbon, G. New regulators of biofilm development in Candida glabrata. *Res. Microbiol.* **2012**, *163*, 297–307.
90. de Groot, P. W. J.; Kraneveld, E. a; Yin, Q. Y.; Dekker, H. L.; Gross, U.; Crielaard, W.; de Koster, C. G.; Bader, O.; Klis, F. M.; Weig, M. The cell wall of the human pathogen Candida glabrata: differential incorporation of novel adhesin-like wall proteins. *Eukaryot. Cell* **2008**, *7*, 1951–64.
91. Kraneveld, E. A.; de Soet, J. J.; Deng, D. M.; Dekker, H. L.; de Koster, C. G.; Klis, F. M.; Crielaard, W.; de Groot, P. W. J. Identification and differential gene expression of adhesin-like wall proteins in Candida glabrata biofilms. *Mycopathologia* **2011**, *172*, 415–27.
92. Silva, S.; Negri, M.; Henriques, M.; Oliveira, R.; Williams, D. W.; Azeredo, J. Adherence and biofilm formation of non-Candida albicans Candida species. *Trends Microbiol.* **2011**, *19*, 241–247.
93. Chupáčová, J.; Borghi, E.; Morace, G.; Los, A.; Bujdáková, H. Anti-biofilm activity of antibody directed against surface antigen CR3-RP - comparison of Candida albicans and Candida dubliniensis. *Pathog. Dis.* **2018**, *76*.
94. Fleming, D.; Rumbaugh, K. P. Approaches to Dispersing Medical Biofilms. *Microorganisms* **2017**, *5*, 15.
95. Silva, S.; Henriques, M.; Oliveira, R.; Williams, D.; Azeredo, J. In Vitro Biofilm Activity of Non-Candida albicans

Candida Species. *Curr. Microbiol.* **2010**, *61*, 534–540.
96. van Dyk, D.; Pretorius, I. S.; Bauer, F. F. Mss11p Is a Central Element of the Regulatory Network That Controls FLO11 Expression and Invasive Growth in Saccharomyces cerevisiae. *Genetics* **2004**, *169*, 91–106.
97. Guo, B.; Styles, C. A.; Feng, Q.; Fink, G. R. A Saccharomyces gene family involved in invasive growth, cell-cell adhesion, and mating. *Proc. Natl. Acad. Sci.* **2000**, *97*, 12158–12163.
98. Cormack, B. P.; Ghori, N.; Falkow, S. An Adhesin of the Yeast Pathogen Candida glabrata Mediating Adherence to Human Epithelial Cells. *Science (80-.).* **1999**, *285*.
99. Sheppard, D. C.; Yeaman, M. R.; Welch, W. H.; Phan, Q. T.; Fu, Y.; Ibrahim, A. S.; Filler, S. G.; Zhang, M.; Waring, A. J.; Edwards, J. E. Functional and Structural Diversity in the Als Protein Family of *Candida albicans*. *J. Biol. Chem.* **2004**, *279*, 30480–30489.
100. Caudle, K. E.; Barker, K. S.; Wiederhold, N. P.; Xu, L.; Homayouni, R.; Rogers, P. D. Genomewide Expression Profile Analysis of the Candida glabrata Pdr1 Regulon. *Eukaryot. Cell* **2011**, *10*, 373–383.
101. Chakrabarti, A.; Nayak, N.; Talwar, P. In vitro proteinase production by Candida species. *Mycopathologia* **1991**, *114*, 163–8.
102. Marcos-Arias, C.; Eraso, E.; Madariaga, L.; Aguirre, J. M.; Quindós, G. Phospholipase and proteinase activities of Candida isolates from denture wearers. *Mycoses* **2011**, *54*, e10–e16.
103. Noble, J. A.; Tsai, H.-F. H.-F.; Suffis, S. D.; Su, Q.; Myers, T. G.; Bennett, J. E. STB5 is a negative regulator of azole resistance in Candida glabrata. *Antimicrob. Agents Chemother.* **2013**, *57*, 959–67.
104. Paul, S.; Bair, T. B.; Moye-Rowley, W. S. Identification of Genomic Binding Sites for Candida glabrata Pdr1 Transcription Factor in Wild-Type and 0 Cells. *Antimicrob. Agents Chemother.* **2014**, *58*, 6904–6912.
105. Paul, S.; Schmidt, J. a; Moye-Rowley, W. S. Regulation of the CgPdr1 transcription factor from the pathogen Candida glabrata. *Eukaryot. Cell* **2011**, *10*, 187–97.
106. Nishikawa, J. L.; Boeszoermenyi, A.; Vale-Silva, L. A.; Torelli, R.; Posteraro, B.; Sohn, Y.-J.; Ji, F.; Gelev, V.; Sanglard, D.; Sanguinetti, M.; Sadreyev, R. I.; Mukherjee, G.; Bhyravabhotla, J.; Buhrlage, S. J.; Gray, N. S.; Wagner, G.; Näär, A. M.; Arthanari, H. Inhibiting fungal multidrug resistance by disrupting an activator-Mediator interaction. *Nature* **2016**, *530*, 485–9.
107. Tsai, H.; Bard, M.; Izumikawa, K.; Krol, A. A.; Sturm, A. M.; Culbertson, N. T.; Pierson, C. A.; Bennett, J. E. Candida glabrata erg1 Mutant with Increased Sensitivity to Azoles and to Low Oxygen Tension. *Antimicrob. Agents Chemother.* **2004**, *48*, 2483–2489.
108. Vermitsky, J.; Edlind, T. D. Azole Resistance in Candida glabrata : Coordinate Upregulation of Multidrug Transporters and Evidence for a Pdr1-Like Transcription Factor. *Antimicrob. Agents Chemother.* **2004**, *48*, 3773–3781.
109. Sanglard, D.; Ischer, F.; Calabrese, D.; Majcherczyk, P. A.; Bille, J. The ATP binding cassette transporter gene CgCDR1 from Candida glabrata is involved in the resistance of clinical isolates to azole antifungal agents. *Antimicrob. Agents Chemother.* **1999**, *43*, 2753–65.
110. Basso, L. R.; Gast, C. E.; Mao, Y.; Wong, B. Fluconazole transport into Candida albicans secretory vesicles by the membrane proteins Cdr1p, Cdr2p, and Mdr1p. *Eukaryot. Cell* **2010**, *9*, 960–970.
111. Paul, S.; Moye-Rowley, W. S. Multidrug resistance in fungi: Regulation of transporter-encoding gene expression. *Front. Physiol.* **2014**, *5 APR*, 143.
112. Szweda, P.; Gucwa, K.; Romanowska, E.; Dzierzanowska-Fangrat, K.; Naumiuk, L.; Brillowska-Dabrowska, A.; Wojciechowska-Koszko, I.; Milewski, S. Mechanisms of azole resistance among clinical isolates of Candida glabrata in Poland. *J. Med. Microbiol.* **2015**, *64*, 610–619.
113. Watamoto, T.; Samaranayake, L. P.; Egusa, H.; Yatani, H.; Seneviratne, C. J. Transcriptional regulation of drug-resistance genes in Candida albicans biofilms in response to antifungals. *J. Med. Microbiol.* **2011**.
114. Kumamoto, C. a. Candida biofilms. *Curr. Opin. Microbiol.* **2002**, *5*, 608–611.
115. Pfaller, M. a Antifungal drug resistance: mechanisms, epidemiology, and consequences for treatment. *Am. J. Med.* **2012**, *125*, S3-13.
116. Singh, A.; Yadav, V.; Prasad, R. Comparative lipidomics in clinical isolates of Candida albicans reveal crosstalk between mitochondria, cell wall integrity and azole resistance. *PLoS One* **2012**, *7*.
117. Halliwell, S. C.; Smith, M. C. a; Muston, P.; Holland, S. L.; Avery, S. V Heterogeneous expression of the virulence-related adhesin Epa1 between individual cells and strains of the pathogen Candida glabrata. *Eukaryot. Cell* **2012**, *11*, 141–50.
118. Juárez-Cepeda, J.; Orta-Zavalza, E.; Cañas-Villamar, I.; Arreola-Gómez, J.; Pérez-Cornejo, G.; Hernández-Carballo, C.; Gutiérrez-Escobedo, G.; Castaño, I.; De Las Peñas, A. The EPA2 adhesin encoding gene is responsive to oxidative stress in the opportunistic fungal pathogen Candida glabrata. *Curr. Genet.* **2015**, *80*.
119. Alves, C. T.; Wei, X. Q.; Silva, S.; Azeredo, J.; Henriques, M.; Williams, D. W. Candida albicans promotes invasion and colonisation of Candida glabrata in a reconstituted human vaginal epithelium. *J. Infect.* **2014**, 1–12.
120. d'Enfert, C.; Janbon, G. Biofilm formation in Candida glabrata: What have we learnt from functional genomics

approaches? *FEMS Yeast Res.* **2016**, *16*, fov111.
121. Borah, S.; Shivarathri, R.; Srivastava, V. K.; Ferrari, S.; Sanglard, D.; Kaur, R. Pivotal role for a tail subunit of the RNA polymerase II mediator complex CgMed2 in azole tolerance and adherence in Candida glabrata. *Antimicrob. Agents Chemother.* **2014**, *58*, 5976–5986.
122. Kaur, R.; Ma, B.; Cormack, B. P. A family of glycosylphosphatidylinositol-linked aspartyl proteases is required for virulence of Candida glabrata. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 7628–33.
123. Tati, S.; Davidow, P.; McCall, A.; Hwang-Wong, E.; Rojas, I. G.; Cormack, B.; Edgerton, M. Candida glabrata Binding to Candida albicans Hyphae Enables Its Development in Oropharyngeal Candidiasis. *PLOS Pathog.* **2016**, *12*, e1005522.
124. Gómez-Molero, E.; de Boer, A. D.; Dekker, H. L.; Moreno-Martínez, A.; Kraneveld, E. A.; Ichsan; Chauhan, N.; Weig, M.; de Soet, J. J.; de Koster, C. G.; Bader, O.; de Groot, P. W. J. Proteomic analysis of hyperadhesive candida glabrata clinical isolates reveals a core wall proteome and differential incorporation of adhesins. *FEMS Yeast Res.* **2015**, *15*.
125. Juárez-Reyes, A.; Ramírez-Zavaleta, C. Y.; Medina-Sánchez, L.; De Las Peñas, A.; Castaño, I. A protosilencer of subtelomeric gene expression in Candida glabrata with unique properties. *Genetics* **2012**, *190*, 101–11.
126. Thompson, G. R.; Wiederhold, N. P.; Vallor, A. C.; Villareal, N. C.; Lewis, J. S.; Patterson, T. F. Development of caspofungin resistance following prolonged therapy for invasive candidiasis secondary to Candida glabrata infection. *Antimicrob. Agents Chemother.* **2008**, *52*, 3783–5.
127. Swoboda-Kopeć, E.; Sikora, M.; Golas, M.; Piskorska, K.; Gozdowski, D.; Netsvyetayeva, I. Candida nivariensis in comparison to different phenotypes of Candida glabrata. *Mycoses* **2014**, *57*, 747–753.
128. Ferrari, S.; Sanguinetti, M.; Torelli, R.; Posteraro, B.; Sanglard, D. Contribution of CgPDR1-regulated genes in enhanced virulence of azole-resistant Candida glabrata. *PLoS One* **2011**, *6*, e17589.
129. Chen, K.-H.; Miyazaki, T.; Tsai, H.-F.; Bennett, J. E. The bZip transcription factor Cgap1p is involved in multidrug resistance and required for activation of multidrug transporter gene CgFLR1 in Candida glabrata. *Gene* **2007**, *386*, 63–72.
130. Farahyar, S.; Zaini, F.; Kordbacheh, P.; Rezaie, S.; Safara, M.; Raoofian, R.; Heidari, M. Overexpression of aldo-keto-reductase in azole-resistant clinical isolates of Candida glabrata determined by cDNA-AFLP. *Daru J. Fac. Pharmacy, Tehran Univ. Med. Sci.* **2013**, *21*, 1.
131. Dudiuk, C.; Gamarra, S.; Leonardeli, F.; Jimenez-Ortigosa, C.; Vitale, R. G.; Afeltra, J.; Perlin, D. S.; Garcia-Effron, G. Set of classical PCRs for detection of mutations in Candida glabrata FKS genes linked with echinocandin resistance. *J. Clin. Microbiol.* **2014**, *52*, 2609–2614.
132. Jensen, R.H., Johansen, H.K. & Arendrup, M. C. Stepwise development of a homozygous S80P substitution in Fks1p, conferring echinocandin resistance in Candida tropicalis. *Antimicrob. Agents Chemother.* **2013**, *57*, 614–617.
133. Saraya, T.; Tanabe, K.; Araki, K.; Yonetani, S.; Makino, H.; Watanabe, T.; Tsujimoto, N.; Takata, S.; Kurai, D.; Ishii, H.; Miyazaki, Y.; Takizawa, H.; Goto, H. Breakthrough Invasive Candida glabrata in Patients on Micafungin: a Novel FKS Gene Conversion Correlated with Sequential Elevation of MIC. *J. Clin. Microbiol.* **2014**, *52*, 2709–2712.
134. Garcia-Effron, G.; Katiyar, S. K.; Park, S.; Edlind, T. D.; Perlin, D. S. A naturally occurring proline-to-alanine amino acid change in Fks1p in Candida parapsilosis, Candida orthopsilosis, and Candida metapsilosis accounts for reduced echinocandin susceptibility. *Antimicrob. Agents Chemother.* **2008**, *52*, 2305–2312.
135. Katiyar, S. K.; Alastruey-Izquierdo, A.; Healey, K. R.; Johnson, M. E.; Perlin, D. S.; Edlind, T. D. Fks1 and Fks2 are functionally redundant but differentially regulated in Candida glabrata: implications for echinocandin resistance. *Antimicrob. Agents Chemother.* **2012**, *56*, 6304–9.
136. Naicker, S. D.; Magobo, R. E.; Zulu, T. G.; Maphanga, T. G.; Luthuli, N.; Lowman, W.; Govender, N. P. Two echinocandin-resistant Candida glabrata FKS mutants from South Africa. *Med. Mycol. Case Rep.* **2016**, *11*, 24–26.
137. Arendrup, M. C.; Perlin, D. S.; Jensen, R. H.; Howard, S. J.; Goodwin, J.; Hope, W. Differential in vivo activities of anidulafungin, caspofungin, and micafungin against Candida glabrata isolates with and without FKS resistance mutations. *Antimicrob. Agents Chemother.* **2012**, *56*, 2435–42.
138. Ostrosky-Zeichner, L. Candida glabrata and FKS mutations: witnessing the emergence of the true multidrug-resistant Candida. *Clin. Infect. Dis.* **2013**, *56*, 1733–4.
139. Pham, C. D.; Iqbal, N.; Bolden, C. B.; Kuykendall, R. J.; Harrison, L. H.; Farley, M. M.; Schaffner, W.; Beldavs, Z. G.; Chiller, T. M.; Park, B. J.; Cleveland, A. a.; Lockhart, S. R. Role of FKS mutations in Candida glabrata: MIC values, echinocandin resistance, and multidrug resistance. *Antimicrob. Agents Chemother.* **2014**, *58*, 4690–4696.
140. Shields, R. K.; Nguyen, M. H.; Press, E. G.; Kwa, A. L.; Cheng, S.; Du, C.; Clancy, C. J. The presence of an FKS mutation rather than MIC is an independent risk factor for failure of echinocandin therapy among patients with invasive candidiasis due to Candida glabrata. *Antimicrob. Agents Chemother.* **2012**, *56*, 4862–9.
141. Perlin, D. S. Mechanisms of echinocandin antifungal drug resistance. *Ann N Y Acad Sci* **2015**, 1–11.
142. Garcia-Effron, G.; Lee, S.; Park, S.; Cleary, J. D.; Perlin, D. S. Effect of Candida glabrata FKS1 and FKS2 mutations

on echinocandin sensitivity and kinetics of 1,3-beta-D-glucan synthase: implication for the existing susceptibility breakpoint. *Antimicrob. Agents Chemother.* **2009**, *53*, 3690–9.
143. Cho, E.-J.; Shin, J. H.; Kim, S. H.; Kim, H.-K.; Park, J. S.; Sung, H.; Kim, M.-N.; Im, H. J. Emergence of multiple resistance profiles involving azoles, echinocandins and amphotericin B in Candida glabrata isolates from a neutropenia patient with prolonged fungaemia. *J. Antimicrob. Chemother.* **2014**, 2–3.
144. Taff, H. T.; Nett, J. E.; Zarnowski, R.; Ross, K. M.; Sanchez, H.; Cain, M. T.; Hamaker, J.; Mitchell, A. P.; Andes, D. R. A Candida biofilm-induced pathway for matrix glucan delivery: implications for drug resistance. *PLoS Pathog.* **2012**, *8*, e1002848.
145. Ibrahim, N. H.; Melake, N. A.; Somily, A. M.; Zakaria, A. S.; Baddour, M. M.; Mahmoud, A. Z. The effect of antifungal combination on transcripts of a subset of drug-resistance genes in clinical isolates of Candida species induced biofilms. *Saudi Pharm. J. SPJ Off. Publ. Saudi Pharm. Soc.* **2015**, *23*, 55–66.
146. Nagahashi, S.; Lussier, M.; Bussey, H. Isolation of Candida glabrata Homologs of the Saccharomyces cerevisiae KRE9 and KNH1 Genes and Their Involvement in Cell Wall ␣1,6-Glucan Synthesis. *J. Bacteriol.* **1998**, *180*, 5020–5029.
147. Vale-Silva, L.; Ischer, F.; Leibundgut-Landmann, S.; Sanglard, D. Gain-of-function mutations in PDR1, a regulator of antifungal drug resistance in Candida glabrata, control adherence to host cells. *Infect. Immun.* **2013**, *81*, 1709–20.
148. Fanning, S.; Mitchell, A. P. Fungal Biofilms. *PLoS Pathog.* **2012**, *8*, e1002585.
149. Schwarzmüller, T.; Ma, B.; Hiller, E.; Istel, F.; Tscherner, M.; Brunke, S.; Ames, L.; Firon, A.; Green, B.; Cabral, V.; Marcet-Houben, M.; Jacobsen, I. D.; Quintin, J.; Seider, K.; Frohner, I.; Glaser, W.; Jungwirth, H.; Bachellier-Bassi, S.; Chauvel, M.; Zeidler, U.; Ferrandon, D.; Gabaldón, T.; Hube, B.; d'Enfert, C.; Rupp, S.; Cormack, B.; Haynes, K.; Kuchler, K. Systematic phenotyping of a large-scale Candida glabrata deletion collection reveals novel antifungal tolerance genes. *PLoS Pathog.* **2014**, *10*, e1004211.
150. Samaranayake, Y. H.; Yau, J. Y. Y.; Thein, Z. M.; Jayatilake, J. A. M. S.; Yeung, K. W. S.; Samaranayake, L. P. The post-antifungal effect (PAFE) of amphotericin B, nystatin, ketoconazole and 5-fluorocytosine and its impact on the colonization traits of Candida glabrata. *Med. Mycol.* **2010**, *48*, 725–34.
151. Nabili, M.; Abdollahi Gohar, A.; Badali, H.; Mohammadi, R.; Moazeni, M. Amino acid substitutions in Erg11p of azole-resistant Candida glabrata: Possible effective substitutions and homology modelling. *J. Glob. Antimicrob. Resist.* **2016**, *5*, 42–46.
152. Vandeputte, P.; Tronchin, G.; Larcher, G.; Ernoult, E.; Bergès, T.; Chabasse, D.; Bouchara, J.-P. A nonsense mutation in the ERG6 gene leads to reduced susceptibility to polyenes in a clinical isolate of Candida glabrata. *Antimicrob. Agents Chemother.* **2008**, *52*, 3701–9.
153. dos Santos Silva, D. B.; Carbonera Rodrigues, L. M.; De Almeida, A. A.; de Oliveira, K. M. P.; Grisolia, A. B. Novel point mutations in the ERG11 gene in clinical isolates of azole resistant Candida species. *Mem. Inst. Oswaldo Cruz* **2016**, *111*, 192–199.
154. Henry, K.; Nickels, J.; Edlind, T. Upregulation of ERG genes in Candida species by azoles and other sterol biosynthesis inhibitors. *Antimicrob Agents Chemother.* **2000**, *44*, 2693–700.
155. Arendrup, M.; Perlin, D.; Jensen, R.; Howard, S.; Goodwin, J.; Hopec, W. Differential in vivo activities of anidulafungin, caspofungin, and micafungin against Candida glabrata isolates with and without FSK resistance mutations. *Antim Agents Chemoter.* **2012**, 2435–2442.
156. Alexander, B. D.; Johnson, M. D.; Pfeiffer, C. D.; Jimenez-Ortigosa, C.; Catania, J.; Booker, R.; Castanheira, M.; Messer, S. A.; Perlin, D. S.; Pfaller, M. A. Increasing echinocandin resistance in Candida glabrata: clinical failure correlates with presence of FKS mutations and elevated minimum inhibitory concentrations. *Clin. Infect. Dis.* **2013**, *56*, 1724–1732.
157. Stead, D. a; Walker, J.; Holcombe, L.; Gibbs, S. R. S.; Yin, Z.; Selway, L.; Butler, G.; Brown, A. J. P.; Haynes, K. Impact of the transcriptional regulator, Ace2, on the Candida glabrata secretome. *Proteomics* **2010**, *10*, 212–23.
158. Maccallum, D. M.; Findon, H.; Kenny, C. C.; Butler, G.; Haynes, K.; Odds, F. C. Different consequences of ACE2 and SWI5 gene disruptions for virulence of pathogenic and nonpathogenic yeasts. *Infect Immun* **2006**, *74*, 5244–8.
159. Kamran, M.; Calcagno, A.; Findon, H.; Bignell, E.; Jones, M. D.; Warn, P.; Hopkins, P.; Denning, D. W.; Butler, G.; Rogers, T.; Mu, F. A.; Haynes, K.; Ace, C. G. Inactivation of Transcription Factor Gene ACE2 in the Fungal Pathogen Candida glabrata Results in Hypervirulence. *Eukaryot. Cell* **2004**, *3*, 546–552.
160. Mulhern, S.; Logue, M.; Butler, G. Candida albicans transcription factor Ace2 regulates metabolism and is required for filamentation in hypoxic conditions. *Eukaryot Cell* **2006**, *5*, 2001–13.
161. Faria-Oliveira, F.; Carvalho, J.; Ferreira, C.; Hernáez, M. L.; Gil, C.; Lucas, C. Quantitative differential proteomics of yeast extracellular matrix: there is more to it than meets the eye. *BMC Microbiol.* **2015**, *15*, 271.
162. Yoo, J. Il; Kim, H. S.; Choi, C. W.; Yoo, J. S.; Yu, J. Y.; Lee, Y. S. Proteomic Analysis of Intracellular and Membrane Proteins From Voriconazole-Resistant Candida glabrata. *Osong Public Heal. Res. Perspect.* **2013**, *4*, 293–300.
163. Kasper, L.; Seider, K.; Gerwien, F.; Allert, S.; Brunke, S.; Schwarzmüller, T.; Ames, L.; Zubiria-Barrera, C.;

Mansour, M. K.; Becken, U.; Barz, D.; Vyas, J. M.; Reiling, N.; Haas, A.; Haynes, K.; Kuchler, K.; Hube, B. Identification of Candida glabrata genes involved in pH modulation and modification of the phagosomal environment in macrophages. *PLoS One* **2014**, *9*, e96015.
164. Pfaller, M. A. a; Castanheira, M.; Lockhart, S. R. R.; Ahlquist, A. M. M.; Messer, S. A. a; Jones, R. N. N. Frequency of decreased susceptibility and resistance to echinocandins among fluconazole-resistant bloodstream isolates of Candida glabrata. *J. Clin. Microbiol.* **2012**, *50*, 1199–1203.
165. Roetzer, A.; Gratz, N.; Kovarik, P.; Schüller, C. Autophagy supports Candida glabrata survival during phagocytosis. *Cell. Microbiol.* **2010**, *12*, 199–216.
166. Cuellar-Cruz, M.; Lopez-Romero, E.; Ruiz-Baca, E.; Zazueta-Sandoval, R. Differential Response of Candida albicans and Candida glabrata to Oxidative and Nitrosative Stresses. *Curr. Microbiol.* **2014**, *69*, 733–739.
167. Alarco, A. M.; Raymond, M. The bZip transcription factor Cap1p is involved in multidrug resistance and oxidative stress response in Candida albicans. *J. Bacteriol.* **1999**, *181*, 700–8.
168. Cuellar-Cruz, M.; Briones-Martin-del-Campo, M.; Canas-Villamar, I.; Montalvo-Arredondo, J.; Riego-Ruiz, L.; Castano, I.; De Las Penas, A. High Resistance to Oxidative Stress in the Fungal Pathogen Candida glabrata Is Mediated by a Single Catalase, Cta1p, and Is Controlled by the Transcription Factors Yap1p, Skn7p, Msn2p, and Msn4p. *Eukaryot. Cell* **2008**, *7*, 814–825.
169. Krems, B.; Charizanis, C.; Entian, K.-D. Mutants of Saccharomyces cerevisiae sensitive to oxidative and osmotic stress. *Curr. Genet.* **1995**, *27*, 427–434.
170. Roetzer, A.; Gregori, C.; Jennings, A. M.; Quintin, J.; Ferrandon, D.; Butler, G.; Kuchler, K.; Ammerer, G.; Schüller, C. Candida glabrata environmental stress response involves Saccharomyces cerevisiae Msn2/4 orthologous transcription factors. *Mol. Microbiol.* **2008**, *69*, 603–20.
171. Zhang, X.; De Micheli, M.; Coleman, S. T.; Sanglard, D.; Moye-Rowley, W. S. Analysis of the oxidative stress regulation of the Candida albicans transcription factor, Cap1p. *Mol. Microbiol.* **2000**, *36*, 618–29.
172. Znaidi, S.; Barker, K. S.; Weber, S.; Alarco, A.-M.; Liu, T. T.; Boucher, G.; Rogers, P. D.; Raymond, M. Identification of the Candida albicans Cap1p Regulon. *Eukaryot. Cell* **2009**, *8*, 806–820.
173. Vandeputte, P.; Tronchin, G.; Bergès, T.; Hennequin, C.; Chabasse, D.; Bouchara, J.-P. Reduced susceptibility to polyenes associated with a missense mutation in the ERG6 gene in a clinical isolate of Candida glabrata with pseudohyphal growth. *Antimicrob. Agents Chemother.* **2007**, *51*, 982–90.
174. Mohan das, V.; Ballal, M. Proteinase and phospholipase activity as virulence factors in Candida species isolated from blood. *Rev. Iberoam. Micol.* **2008**, *25*, 208–10.
175. Kalkanci, A.; GüZel, A. B.; Khalil, I. I. J.; Aydin, M.; Ilkit, M.; Kuştimur, S. Yeast vaginitis during pregnancy: susceptibility testing of 13 antifungal drugs and boric acid and the detection of four virulence factors. *Med. Mycol.* **2012**, *50*, 585–593.
176. Ueno, K.; Matsumoto, Y.; Uno, J.; Sasamoto, K.; Sekimizu, K.; Kinjo, Y.; Chibana, H. Intestinal resident yeast Candida glabrata requires Cyb2p-Mediated lactate assimilation to adapt in mouse intestine. *PLoS One* **2011**, *6*.
177. Sikora, M.; Dabkowska, M.; Swoboda-Kopec, E.; Jarzynka, S.; Netsvyetayeva, I.; Jaworska-Zaremba, M.; Pertkiewicz, M.; Mlynarczyk, G. Differences in proteolytic activity and gene profiles of fungal strains isolated from the total parenteral nutrition patients. *Folia Microbiol. (Praha).* **2011**, *56*, 143–148.
178. Negri, M.; Martins, M.; Henriques, M.; Svidzinski, T. I. E.; Azeredo, J.; Oliveira, R. Examination of Potential Virulence Factors of Candida tropicalis Clinical Isolates From Hospitalized Patients. *Mycopathologia* **2010**, *169*, 175–182.
179. Luo, G.; Samaranayake, L. P. Candida glabrata, an emerging fungal pathogen, exhibits superior relative cell surface hydrophobicity and adhesion to denture acrylic surfaces compared with Candida albicans. *APMIS* **2002**, *110*, 601–10.
180. LUO, G.; SAMARANAYAKE, L. P.; CHEUNG, B. P. K.; TANG, G. Reverse transcriptase polymerase chain reaction (RT-PCR) detection of HLP gene expression in Candida glabrata and its possible role in in vitro haemolysin production. *APMIS* **2004**, *112*, 283–290.
181. Berila, N.; Hyroššová, P.; Subík, J. Oxidative stress response and virulence factors in Candida glabrata clinical isolates. *Folia Microbiol. (Praha).* **2011**, *56*, 116–21.
182. Roetzer, A.; Gabaldón, T.; Schüller, C. From Saccharomyces cerevisiae to Candida glabratain a few easy steps: important adaptations for an opportunistic pathogen. *FEMS Microbiol. Lett.* **2011**, *314*, 1–9.
183. Kramer, A.; Schwebke, I.; Kampf, G. How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. *BMC Infect. Dis.* **2006**, *6*, 130.
184. Cox, G. M.; Harrison, T. S.; McDade, H. C.; Taborda, C. P.; Heinrich, G.; Casadevall, A.; Perfect, J. R. Superoxide dismutase influences the virulence of Cryptococcus neoformans by affecting growth within macrophages. *Infect. Immun.* **2003**, *71*, 173–80.
185. Nicola, A. M.; Casadevall, A.; Goldman, D. L. Fungal killing by mammalian phagocytic cells. *Curr. Opin. Microbiol.* **2008**, *11*, 313–7.

186. Segal, A. W. How neutrophils kill microbes. *Annu. Rev. Immunol.* **2005**, *23*, 197–223.
187. Nakagawa, Y.; Kanbe, T.; Mizuguchi, I. Disruption of the human pathogenic yeast Candida albicans catalase gene decreases survival in mouse-model infection and elevates susceptibility to higher temperature and to detergents. *Microbiol. Immunol.* **2003**, *47*, 395–403.
188. Jacobsen, I. D.; Brunke, S.; Seider, K.; Schwarzmüller, T.; Firon, A.; D 'enfért, C.; Kuchler, K.; Hube, B. Candida glabrata Persistence in Mice Does Not Depend on Host Immunosuppression and Is Unaffected by Fungal Amino Acid Auxotrophy. *Infect. Immun.* **2010**, *78*, 1066–1077.
189. Schaller, M.; Sander, C. A.; Korting, H. C.; Mailhammer, R.; Grassl, G.; Hube, B. Infection of Human Oral Epithelia with Candida Species Induces Cytokine Expression Correlated to the Degree of Virulence. *J. Invest. Dermatol.* **2002**, *118*, 652–657.
190. Li, L.; Kashleva, H.; Dongari-Bagtzoglou, A. Cytotoxic and cytokine inducing properties of Candida glabrata in single and mixed oral infection models.
191. Filler, S. G.; Swerdloff, J. N.; Hobbs, C.; Luckett, P. M. Penetration and Damage of Endothelial Cells by Candida albicans. *Infect. Immun.* **1995**, *63*, 976–983.
192. Netea, M. G.; Joosten, L. A.; van der Meer, J. W.; Kullberg, B. J.; van de Veerdonk, F. L. Immune defence against Candida fungal infections. *Nat Rev Immunol* **2015**, *15*.
193. Shoham, S.; Levitz, S. M. The immune response to fungal infections. *Br. J. Haematol.* **2005**, *129*, 569–582.
194. V Zquez-torres, A. S.; Balish, E. Macrophages in Resistance to Candidiasis. *Microbiol. Mol. Biol. Rev.* **1997**, *61*, 170–192.
195. Wagner, R. D.; Vazquez-Torres, A.; Jones-Carson, J.; Warner, T.; Balish, E. B Cell Knockout Mice Are Resistant to Mucosal and Systemic Candidiasis of Endogenous Origin but Susceptible to Experimental Systemic Candidiasis. *J. Infect. Dis.* **1996**, *174*.
196. Westwater, C.; Schofield, D. A.; Nicholas, P. J.; Paulling, E. E.; Balish, E. *Candida glabrata* and *Candida albicans* ; dissimilar tissue tropism and infectivity in a gnotobiotic model of mucosal candidiasis. *FEMS Immunol. Med. Microbiol.* **2007**, *51*, 134–139.
197. Keppler-Ross, S.; Douglas, L.; Konopka, J. B.; Dean, N. Recognition of Yeast by Murine Macrophages Requires Mannan but Not Glucan. *Eukaryot. Cell* **2010**, *9*, 1776–1787.
198. Seider, K.; Brunke, S.; Schild, L.; Jablonowski, N.; Wilson, D.; Majer, O.; Dagmar, B.; Haas, A.; Kuchler, K.; Schaller, M.; Huber, B. The Facultative Intracellular Pathogen Candida glabrata Subverts Macrophage Cytokine Production and Phagolysosome Maturation. *J. Immunol.* **2011**, *187*, 3072–3086.
199. Klionsky, D. J. Cell biology: Regulated self-cannibalism. *Nature* **2004**, *431*, 31–32.
200. Klionsky, D. J. Autophagy. *Curr. Biol.* **2005**, *15*, R282–R283.
201. Xie, Z.; Klionsky, D. J. Autophagosome formation: core machinery and adaptations. *Nat. Cell Biol.* **2007**, *9*, 1102–1109.
202. Saijo, T.; Miyazaki, T.; Izumikawa, K.; Mihara, T.; Takazono, T.; Kosai, K.; Imamura, Y.; Seki, M.; Kakeya, H.; Yamamoto, Y.; Yanagihara, K.; Kohno, S. Skn7p Is Involved in Oxidative Stress Response and Virulence of Candida glabrata. *Mycopathologia* **2010**, *169*, 81–90.
203. Lee, J.; Godon, C.; Lagniel, G.; Spector, D.; Garin, J.; Labarre, J.; Toledano, M. B. Yap1 and Skn7 control two specialized oxidative stress response regulons in yeast. *J. Biol. Chem.* **1999**, *274*, 16040–6.
204. Gulshan, K.; Lee, S. S.; Moye-Rowley, W. S. Differential Oxidant Tolerance Determined by the Key Transcription Factor Yap1 Is Controlled by Levels of the Yap1-binding Protein, Ybp1. *J. Biol. Chem.* **2011**, *286*, 34071–34081.
205. Roetzer, A.; Klopf, E.; Gratz, N.; Marcet-Houben, M.; Hiller, E.; Rupp, S.; Gabaldón, T.; Kovarik, P.; Schüller, C. Regulation of *Candida glabrata* oxidative stress resistance is adapted to host environment. *FEBS Lett.* **2011**, *585*, 319–327.
206. Lorenz, M. C.; Bender, J. A.; Fink, G. R. Transcriptional Response of Candida albicans upon Internalization by Macrophages. *Eukaryot. Cell* **2004**, *3*, 1076–1087.
207. Verstrepen, K. J.; Jansen, A.; Lewitter, F.; Fink, G. R. Intragenic tandem repeats generate functional variability. *Nat. Genet.* **2005**, *37*, 986–990.
208. Ruan, S.-Y.; Hsueh, P.-R. Invasive Candidiasis: An Overview from Taiwan. *J. Formos. Med. Assoc.* **2009**, *108*, 443–451.
209. Domergue, R.; Castaño, I.; De Las Peñas, A.; Zupancic, M.; Lockatell, V.; Hebel, J. R.; Johnson, D.; Cormack, B. P. Nicotinic Acid Limitation Regulates Silencing of Candida Adhesins During UTI. *Science (80-.).* **2005**, *308*, 866–870.
210. Castaño, I.; Pan, S.-J.; Zupancic, M.; Hennequin, C.; Dujon, B.; Cormack, B. P. Telomere length control and transcriptional regulation of subtelomeric adhesins in Candida glabrata. *Mol. Microbiol.* **2004**, *55*, 1246–1258.
211. Jandric, Z.; Schuller, C. Stress response in Candida glabrata: pieces of a fragmented picture. *Futur. Mirobiology* **2011**, *6*, 1475–1484.
212. Silva, S.; Henriques, M.; Hayes, A.; Oliveira, R.; Azeredo, J.; Williams, D. W. Candida glabrata and Candida

albicans co-infection of an in vitro oral epithelium. *J. Oral Pathol. Med.* **2011**, *40*, 421–427.
213. Ramage, G.; Rajendran, R.; Sherry, L.; Williams, C. Fungal biofilm resistance. *Int. J. Microbiol.* **2012**, *2012*, 528521.
214. Alcazar- Fuoli, L.; Mellado, E. Current status of antifungal resistance and its impact on clinical practice. *Br. J. Haematol.* **2014**, *166*.
215. ESCMID Study Group for Biofilms (ESGB) EUCAST definitions (and breakpoint table, MIC and zone distribution website conventions) http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/EUCAST_Presentations/2011/EW1_Brown_Definitionsf2.pdf.
216. Akins, R. A. An update on antifungal targets and mechanisms of resistance in Candida albicans. *Med. Mycol.* **2005**, *43*, 285–318.
217. Mathé, L.; Van Dijck, P. Recent insights into Candida albicans biofilm resistance mechanisms. *Curr. Genet.* **2013**, *59*, 251–264.
218. Taff, H. T.; Mitchell, K. F.; Edward, J. A.; Andes, D. R. Mechanisms of Candida biofilm drug resistance. *Futur. Microbiol.* **2013**, *8*, 1325–1337.
219. Fox, E.; Singh-babak, S.; Hartooni, N.; Nobile, C. Biofilms and antifungal resistance. In *Antifungals from genomics to resistance and the development of novel agents*; Coste, A.; Vandeputte, P., Eds.; Caister Academic Press, 2015; pp. 71–90.
220. Anderson, J. B. Evolution of antifungal-drug resistance: mechanisms and pathogen fitness. *Nat. Rev. Microbiol.* **2005**, *3*, 547–556.
221. Jabra-Rizk, M. A. M. A.; Falkler, W. A. W. a.; Meiller, T. F. T. F. Fungal Biofilms and Drug Resistance. *Emerg. Infect. Dis.* **2004**, *10*, 14–19.
222. Nett, J.; Lepak, A.; Marchillo, K.; Andes, D. Time course global gene expression analysis of an in vivo Candida biofilm. *J Infect Dis* **2009**, *200*, 307–313.
223. Ramage, G.; Vandewalle, K.; Wickes, B.; López-Ribot, J. Characteristics of biofilm formation by Candida albicans. *Rev. Iberoam. Micol.* **2001**, *18*, 163–170.
224. Mukherjee, P. K.; Chandra, J.; Kuhn, D. M.; Ghan- noum, M. A. Mechanism of fluconazole resistance in Candida albicans biofilms: phase-specific role of efflux pumps and membrane sterols. *Infect. Immun.* **2003**, *71*, 4333–4340.
225. Andes, D.; Nett, J.; Oschel, P.; Albrecht, R.; Marchillo, K.; Pitula, A. Development and characterization of an in vivo central venous catheter Candida albicans biofilm model. *Infect. Immun.* **2004**, *72*, 6023–6031.
226. Mateus, C.; Crow, S. A.; Ahearn, D. G. Adherence of Candida albicans to silicone induces immediate enhanced tolerance to fluconazole. *Antimicrob. Agents Chemother.* **2004**, *48*, 3358–3366.
227. Nobile, C. J.; Fox, E. P.; Nett, J. E.; Sorrells, T. R.; Mitrovich, Q. M.; Hernday, A. D.; Tuch, B. B.; Andes, D. R.; Johnson, A. D. A recently evolved transcriptional network controls biofilm development in Candida albicans. *Cell* **2012**, *148*, 126–38.
228. Ramage, G.; Bachmann, S.; Patterson, T. F.; Wickes, B. L.; López-Ribot, J. L. Investigation of multidrug efflux pumps in relation to fluconazole resistance in Candida albicans biofilms. *J. Antimicrob. Chemother.* **2002**, *49*, 973–980.
229. Yeater, K.; Chandra, J.; Cheng, G.; Mukherjee, P.; Zhao, X.; Rodriguez- Zas, S.; Kwast, K.; Ghannoum, M.; Hoyer, L. Temporal analysis of Candida albicans gene expression during biofilm development. *Microbiology* **2007**, 2373–2385.
230. Nguyen, M. H.; Yu, C. Y. Influence of incubation time, inoculum size, and glucose concentrations on spectrophotometric endpoint determinations for amphotericin B, fluconazole, and itraconazole. *J. Clin. Microbiol.* **1999**, *37*, 141–145.
231. *European Committee on Antimicrobial Susceptibility Testing – Antifungal agents, Breakpoint tables for interpretation of MICs, Version 8.0, valid from 2015-11-16*.
232. *National Committee for Clinical Laboratory Standards*; 1997.
233. Perumal, P.; Mekala, S.; Chaffin, W. L. Role for cell density in antifungal drug resistance in Candida albicans biofilms. *Antimicrob. Agents Chemother.* **2007**, *51*, 2454–2463.
234. Cannon, R.; Lamping, E.; Holmes, A. Candida albicans drug resistance—another way to cope with stress. *Microbiol* **2007**, *153*, 3211–3217.
235. Costa-Orlandi, C.; Sardi, J.; Pitangui, N.; de Oliveira, H.; Scorzoni, L.; Galeane, M.; Medina-Alarcón, K.; Melo, W.; Marcelino, M.; Braz, J.; Fusco-Almeida, A.; Mendes-Giannini, M. Fungal Biofilms and Polymicrobial Diseases. *J. Fungi* **2017**, *3*, 22.
236. Fox, E. P.; Singh-babak, S. D.; Hartooni, N.; Nobile, C. J. Biofilms and Antifungal Resistance. In; 2013.
237. Cuéllar-Cruz, M.; López-Romero, E.; Ruiz-Baca, E.; Zazueta-Sandoval, R. Differential Response of Candida albicans and Candida glabrata to Oxidative and Nitrosative Stresses. *Curr. Microbiol.* **2014**, *69*, 733–739.
238. Kasper, L.; Seider, K.; Gerwien, F.; Allert, S.; Brunke, S.; Schwarzmüller, T.; Ames, L.; Zubiria-Barrera, C.;

Mansour, M. K.; Becken, U.; Barz, D.; Vyas, J. M.; Reiling, N.; Haas, A.; Haynes, K.; Kuchler, K.; Hube, B. Identification of Candida glabrata Genes Involved in pH Modulation and Modification of the Phagosomal Environment in Macrophages. *PLoS One* **2014**, *10*, 1–11.
239. Chandra, J.; Kuhn, D.; Mukherjee, P.; Hoyer, L.; McCormick, T.; Ghannoum, M. Biofilm formation by the fungal pathogen Candida albicans: development, architecture, and drug resistance. *J Bacteriol* **2001**, *183*, 5385–5394.
240. Hawser, S. Adhesion of different Candida spp. to plastic: XTT formazan determinations. *J. Med. Vet. Mycol. 34*, 407–10.
241. Kuhn, D. M.; Balkis, M.; Chandra, J.; Mukherjee, P. K.; Ghannoum, M. A. Uses and Limitations of the XTT Assay in Studies of Candida Growth and Metabolism. *J. Clin. Microbiol.* **2003**, *41*, 506.
242. E, M.; Lang S; C, W.; E, M.; B, J.; et al Phase-dependent antifungal activity against Aspergillus fumigatus developing multicellular filamentous biofilms. *J Antimicrob Chemother* **2008**, *62*, 1281–1284.
243. Kucharíková, S.; Iè ne Tournu, H.; Lagrou, K.; Van Dijck, P.; Bujdá ková, H.; Helena Bujdá ková, C. Detailed comparison of Candida albicans and Candida glabrata biofilms under different conditions and their susceptibility to caspofungin and anidulafungin.
244. Pettit, R.; Repp, K.; Hazen, K. Temperature affects the susceptibility of Cryptococcus neoformans biofilms to antifungal agents. *Med. Mycol.* **2009**, *48*, 1–7.
245. Kean, R.; Delaney, C.; Rajendran, R.; Sherry, L.; Metcalfe, R.; Thomas, R.; McLean, W.; Williams, C.; Ramage, G. Gaining Insights from Candida Biofilm Heterogeneity: One Size Does Not Fit All. *J. Fungi* **2018**, *4*, 12.
246. Cortez-Sánchez, J. L.; Cortés-Acosta, E.; Cueto-Hernández, V. M.; Reyes-Maldonado, E.; Hernández-Rodríguez, C.; Villa-Tanaca, L.; Ibarra, J. A. Activity and expression of Candida glabrata vacuolar proteases in autophagy-like conditions. *FEMS Yeast Res.* **2018**.
247. Balzi, E.; Goffeau, A. Yeast multidrug resistance: the PDR network. *J. Bioenerg. Biomembr.* **1995**, *27*, 71–6.
248. Higgins, C. F. ABC Transporters: From Microorganisms to Man. *Annu. Rev. Cell Biol.* **1992**, *8*, 67–113.
249. Albertson, G. D.; Niimi, M.; Cannon, R. D.; Jenkinson, H. F. Multiple efflux mechanisms are involved in Candida albicans fluconazole resistance. *Antimicrob. Agents Chemother.* **1996**, *40*, 2835–41.
250. Lopez-Ribot, J. L.; McAtee, R. K.; Perea, S.; Kirkpatrick, W. R.; Rinaldi, M. G.; Patterson, T. F. Multiple resistant phenotypes of Candida albicans coexist during episodes of oropharyngeal candidiasis in human immunodeficiency virus-infected patients. *Antimicrob. Agents Chemother.* **1999**, *43*, 1621–30.
251. Sanglard, D.; Ischer, F.; Monod2, M.; Billel, J. Cloning of Candida albicans genes conferring resistance to azole antifungal agents: characterization of CDR2, a new multidrug ABC transporter gene. *Microbiology 143*, 405–41.
252. Parkinson, T.; Falconer, D. J.; Hitchcock, C. A. Fluconazole resistance due to energy-dependent drug efflux in Candida glabrata. *Antimicrob. Agents Chemother.* **1995**, *39*, 1696–9.
253. Lepak, A.; Nett, J.; Lincoln, L.; Marchillo, K.; Andes, D. Time Course of Microbiologic Outcome and Gene Expression in Candida albicans during and following In Vitro and In Vivo Exposure to Fluconazole. *Antimicrob. Agents Chemother.* **2006**, *50*, 1311–1319.
254. Perlin, D. S.; Rautemaa-Richardson, R.; Alastruey-Izquierdo, A. Series Fungal infections 5 The global problem of antifungal resistance: prevalence, mechanisms, and management. *Lancet Infect. Dis.* **2017**, *3099*, e383–e392.
255. Ramage, G.; Mowat, E.; Jones, B.; Williams, C.; Lopez-Ribot, J. Our Current Understanding of Fungal Biofilms. *Crit. Rev. Microbiol.* **2009**, *35*, 340–355.
256. Alnuaimi, A. D.; O'Brien-Simpson, N. M.; Reynolds, E. C.; Mccullough, M. J. Clinical isolates and laboratory reference Candida species and strains have varying abilities to form biofilms. *FEMS Yeast Res.* **2013**, *13*.
257. Wang, Z.; Shen, Y.; Haapasalo, M. Antibiofilm peptides against oral biofilms. *J. Oral Microbiol.* **2017**, *9*.
258. Johnson, C. J.; Kernien, J. F.; Hoyer, A. R.; Nett, J. E. Mechanisms involved in the triggering of neutrophil extracellular traps (NETs) by Candida glabrata during planktonic and biofilm growth.
259. Fidel, P. L.; Vazquez, J. A.; Sobel, J. Candida glabrata: Review of Epidemiology, Pathogenesis, and Clinical Disease with Comparison to C. albicans. *Clin. Microbiol. Rev.* **1999**, *12*, 80–96.
260. Morio, F.; Jensen, R. H.; Pape, P. Le; Arendrup, M. C. Molecular basis of antifungal drug resistance in yeasts. *Int. J. Antimicrob. Agents* **2017**, *17*.
261. Al-Dhaheri, R. S. R. S.; Douglas, L. J. J. Absence of amphotericin B-tolerant persister cells in biofilms of some Candida species. *Antimicrob. Agents Chemother.* **2008**, *52*, 1884–1887.
262. LaFleur, M. D.; Kumamoto, C. A.; Lewis, K. Candida albicans biofilms produce antifungal-tolerant persister cells. *Antimicrob. Agents Chemother.* **2006**, *50*, 3839–3846.
263. Fauvart, M.; De Groote, V. N.; Michiels, J. Role of persister cells in chronic infections: clinical relevance and perspectives on anti-persister therapies. *J. Med. Microbiol.* **2011**, *60*, 699–709.
264. Delarze, E.; Sanglard, D. Defining the frontiers between antifungal resistance, tolerance and the concept of persistence. *Drug Resist. Updat.* **2015**, *23*.
265. Dawson, C. C.; Intapa, C.; Jabra-Rizk, M. A. "Persisters": Survival at the cellular level. *PLoS Pathog.* **2011**, *7*, 2–4.

266. Lewis K Multidrug tolerance of biofilms and persister cells. *Cur T Microbiol Immunol* **2008**, *322*, 7–131.
267. Hornby, J.; Jensen, E.; Lisec, A.; Tasto, J.; Jahnke, B.; Shoemaker, R.; Dussault, P.; Nickerson, K. Quorum sensing in the dimorphic fungus Candida albicans is mediated by farnesol. *Appl Env. Microbiol* **2001**, *67*, 2982–2992.
268. Al-fattani, M. a; Douglas, L. J. Penetration of Candida Biofilms by Antifungal Agents. *Antimicrob Agents Chemother* **2004**, *48*, 3291–3297.
269. Al-Fattani, M. A.; Douglas, L. J. Biofilm matrix of Candida albicans and Candida tropicalis: Chemical composition and role in drug resistance. *J. Med. Microbiol.* **2006**, *55*, 999–1008.
270. Lal, P.; Sharma, D.; Pruthi, P.; Pruthi, V. Exopolysaccharide analysis of biofilm-forming Candida albicans. *J. Appl. Microbiol.* **2010**, *109*, 128–136.
271. Martins, M.; Uppuluri, P.; Thomas, D. P.; Cleary, I. A.; Henriques, M.; Lopez-Ribot, J. L.; Oliveira, R. Presence of extracellular DNA in the Candida albicans biofilm matrix and its contribution to biofilms. *Mycopathologia* **2010**, *169*, 323–331.
272. Martins, M.; Henriques, M.; Lopez-Ribot, J. L.; Oliveira, R. Addition of DNase improves the in vitro activity of antifungal drugs against Candida albicans biofilms. *Mycoses* **2012**, *55*, 80–85.
273. Kojic, E. M. E. M.; Darouiche, R. O. R. O. Candida infections of medical devices. *Clin Microbiol Rev.* **2004**, *17*, 255–267.
274. Mitchell, K. F.; Taff, H. T.; Cuevas, M. A.; Reinicke, E. L.; Sanchez, H.; Andes, D. R. Role of matrix β-1,3-glucan in antifungal resistance of non-albicans Candida biofilms. *Antimicrob. Agents Chemother.* **2013**, *57*, 1918–1920.
275. Vediyappan, G.; Rossignol, T.; D'Enfert, C. Interaction of Candida albicans biofilms with antifungals: Transcriptional response and binding of antifungals to beta-glucans. *Antimicrob. Agents Chemother.* **2010**.
276. Nett, J. E.; Crawford, K.; Marchillo, K.; Andes, D. R.; Nett, J.E., Crawford, K., Marchillo, K., and Andes, D. R. Role of Fks1p and matrix glucan in Candida albicans biofilm resistance to an echinocandin, pyrimidine, and polyene. *Antimicrob. Agents Chemother.* **2010**, *54*, 3505–3508.
277. Bandara, H. M. H. N.; Lam, O. L. T.; Watt, R. M.; Jin, L. J.; Samaranayake, L. P. Bacterial lipopolysaccharides variably modulate in vitro biofilm formation of Candida species. *J. Med. Microbiol.* **2010**, *59*, 1225–34.
278. Zhu Z, Wang H, Shang Q, Jiang Y, Cao Y, C. Y. Time course analysis of Candida albicans metabolites during biofilm development. *J Proteome Res* **2012**, *12*, 2375–2385.
279. Baillie, G. S.; Douglas, L. J. Effect of growth rate on resistance of Candida albicans biofilms to antifungal agents. *Antimicrob. Agents Chemother.* **1998**, *42*, 1900–1905.
280. Baillie, G. S.; Douglas, L. J. Iron-limited biofilms of Candida albicans and their susceptibility to amphotericin B. *Antimicrob. Agents Chemother.* **1998**, *42*, 2146–2149.
281. Seneviratne, C. J.; Wang, Y.; Jin, L.; Abiko, Y.; Samaranayake, L. P.; Jayampath Seneviratne, C.; Wang, Y.; Jin, L.; Abiko, Y.; Samaranayake, L. P. Proteomics of drug resistance in candida glabrata biofilms. *Proteomics* **2010**, *10*, 1444–1454.
282. Khot, P. D.; Suci, P. A.; Miller, R. L.; Nelson, R. D.; Tyler, B. J. A small subpopulation of blastospores in Candida albicans biofilms exhibit resistance to amphotericin B associated with differential regulation of ergosterol and beta-1,6-glucan pathway genes. *Antimicrob. Agents Chemother.* **2006**, *50*, 3708–3716.
283. LaFleur, M.; Qingguo, Q.; Lewis, K. Patients with long-term oral carriage harbor high-persister mutants of Candida albicans. *Antimicrob. Agents Chemother.* **2010**, *54*, 39–44.
284. Lewis, K. Persister cells. *Annu. Rev. Microbiol.* **2010**, *64*, 357–372.
285. Bojsen, R.; Regenberg, B.; Gresham, D.; Folkesson, A. A common mechanism involving the TORC1 pathway can lead to amphotericin B-persistence in biofilm and planktonic Saccharomyces cerevisiae populations. *Sci. Rep.* **2016**, *6*, 21874.
286. Niimi, M.; Firth, N. A.; Cannon, R. D. Antifungal drug resistance of oral fungi. *Odontology* 2010.
287. Cleveland, A. A.; Farley, M. M.; Harrison, L. H.; Stein, B.; Hollick, R.; Lockhart, S. R.; Magill, S. S.; Derado, G.; Park, B. J.; Chiller, T. M. Changes in incidence and antifungal drug resistance in candidemia: results from pop- ulation-based laboratory surveillance in Atlanta and Baltimore, 2008–2011. *Clin. Infect. Dis.* **2012**, *55*, 1352–1361.
288. Shahi, P.; Moye-Rowley, W. S. Coordinate control of lipid composition and drug transport activities is required for normal multidrug resistance in fungi. *Biochim. Biophys. Acta* **2009**, *1794*, 852–9.
289. Muller, H.; Thierry, A.; Coppée, J.-Y.; Gouyette, C.; Hennequin, C.; Sismeiro, O.; Talla, E.; Dujon, B.; Fairhead, C. Genomic polymorphism in the population of Candida glabrata: gene copy-number variation and chromosomal translocations. *Fungal Genet. Biol.* **2009**, *46*, 264–76.
290. Thierry, A.; Bouchier, C.; Dujon, B.; Richard, G.-F. Megasatellites: a peculiar class of giant minisatellites in genes involved in cell adhesion and pathogenicity in Candida glabrata. *Nucleic Acids Res.* **2008**, *36*, 5970–82.
291. Barchiesi, F.; Falconi Di Francesco, L.; Arzeni, D.; Caselli, F.; Gallo, D.; Scalise, G. Electrophoretic Karyotyping and Triazole Susceptibility of Candida glabrata Clinical Isolates. *Eur. J. Clin. Microbiol. Infect. Dis.* **1999**, *18*, 184–187.

292. Kaufmann, C. S.; Merz, W. G. Electrophoretic Karyotypes of Torulopsis glabrata. *J. Clin. Microbiol.* **1989**, *27*, 2165–2168.
293. Klempp-Selb, B.; Rimek, D.; Kappe, R. Karyotyping of Candida albicans and Candida glabrata from patients with Candida sepsis. *Mycoses* **2000**, *43*, 159–63.
294. Lin, C.-Y.; Chen, Y.-C.; Lo, H.-J.; Chen, K.-W.; Li, S.-Y. Assessment of Candida glabrata Strain Relatedness by Pulsed-Field Gel Electrophoresis and Multilocus Sequence Typing. *J. Clin. Microbiol.* **2007**, *45*, 2452–2459.
295. Shin, J. H.; Chae, M. J.; Song, J. W.; Jung, S.-I.; Cho, D.; Kee, S. J.; Kim, S. H.; Shin, M. G.; Suh, S. P.; Ryang, D. W. Changes in karyotype and azole susceptibility of sequential bloodstream isolates from patients with Candida glabrata candidemia. *J. Clin. Microbiol.* **2007**, *45*, 2385–91.
296. Magee, B. B.; Sanchez, M. D.; Saunders, D.; Harris, D.; Berriman, M.; Magee, P. T. Extensive chromosome rearrangements distinguish the karyotype of the hypovirulent species Candida dubliniensis from the virulent Candida albicans. *Fungal Genet. Biol.* **2008**, *45*, 338–50.
297. Bader, O.; Schwarz, A.; Kraneveld, E. A.; Tangwattanchuleeporn, M.; Schmidt, P.; Jacobsen, M. D.; Gross, U.; De Groot, P. W. J.; Weig, M. Gross Karyotypic and Phenotypic Alterations among Different Progenies of the Candida glabrata CBS138/ ATCC2001 Reference Strain. *PLoS One* **2012**, *7*, e52218.
298. Rai, M. N.; Balusu, S.; Gorityala, N.; Dandu, L.; Kaur, R. Functional genomic analysis of Candida glabrata-macrophage interaction: role of chromatin remodeling in virulence. *PLoS Pathog.* **2012**, *8*, e1002863.
299. Lemke, A.; Kiderlen, A. F.; Kayser, O. Amphotericin B. *Appl. Microbiol. Biotechnol.* **2005**, *68*, 151–162.
300. Baginski, M.; Czub, J. Amphotericin B and its new derivatives-mode of action. *Curr Drug Metab* **2009**, *10*, 459–69.
301. Botero, M. C.; Puentes-Herrera, M.; Cortés, J. A. Formas lipídicas de anfotericina. *Rev Chil. Infectol* **2014**, *31*, 518–527.
302. Perlin, D. S. Resistance to echinocandin- class antifungal drugs. *Drug Resist. Updat.* **2007**, *10*, 121–130.
303. Debono, M.; Gordee, R. S. S. Antibiotics that inhibit fungal cell wall development. *Annu. Rev. Microbiol.* **1994**, *48*, 471–497.
304. Van Bambeke, François; Balzi, Elisabetta; Tulkens, P. M. Antibiotic Efflux Pumps. *Biochem. Pharmacol.* **2000**, *60*, 457–470.
305. Canuto, M. M.; Rodero, F. G. Antifungal drug resistance to azoles and polyenes. *Lancet Infect. Dis.* **2002**, *2*, 550–563.
306. Maenza, J.; Merz, W.; Romagnoli, M.; Keruly, J.; Moore, R.; Gallant, J. Infection due to fluconazole- resistant Candida in patients with AIDS: prevalence and microbiology. *Clin Infect Dis* **1997**, *24*, 28–34.
307. Laguna, F.; Rodríguez-Tudela, J. L.; Martínez-Súarez, J. V; Polo, R.; Valencia, E.; Díaz-Guerra, T. M.; Dronda, F.; Pulido, F.; Martínez-Suárez, J. Patterns of fluconazole susceptibility in isolates from human immunodeficiency virus-infected patients with oropharyngeal candidiasis due to Candida albicans. *Clin Infect Dis* **1997**, *24*, 124–30.
308. Revankar, S. G.; Kirkpatrick, W. R.; McAtee, R. K.; Dib, O. P.; Fothergill, A. W.; Redding, S. W.; Rinaldi, M. G.; Patterson, T. F. Detection and significance of fluconazole resistance in oropharyngeal candidiasis in human immunodeficiency virus-infected patients. *J Infect Dis* **1996**, *174*, 821–27.
309. Wingard, J.; Merz, W.; Rinaldi, M.; Jonhson, T.; Karp, J.; Saral, R. Increase in Candida krusei infection among patients with bone marrow transplantation and neutropenia treated prophylactically with fluconazole. *N Engl J Med* **1991**, *325*, 1274–77.
310. Gutiérrez, F.; Wall, P.; Cohen, J. Trends in antifungal use and antifungal isolates in a university hospital 1990–1992. *J Hosp Infect* **1995**, *31*, 149–52.
311. Nguyen, M. L. H.; Peacock, J. E.; Morris, A. J.; Tanner, D. C.; Nguyen, M. L. H.; Snydman, D. R.; Wagener, M. M.; Rinaldi, M. G.; Yu, V. L. The changing face of candidemia: emergence of non- Candida albicans species and antifungal resistance. *Am J Med* **1996**, *100*, 617–23.
312. Wilson, D.; Thewes, S.; Zakikhany, K.; Fradin, C.; Albrecht, A.; Almeida, R.; Brunke, S.; Grosse, K.; Martin, R.; Mayer, F.; Leonhardt, I.; Schild, L.; Seider, K.; Skibbe, M.; Slesiona, S.; Waechtler, B.; Jacobsen, I.; Hube, B. Identifying infection-associated genes of Candida albicans in the postgenomic era. *FEMS Yeast Res.* **2009**, *9*, 688–700.
313. Brunke, S.; Seider, K.; Almeida, R.; Heyken, A.; Fleck, C.; Brock, M.; Barz, D.; Rupp, S.; Hube, B. Candida glabrata tryptophan-based pigment production via the Ehrlich pathway. *Mol. Microbiol.* **2010**, *76*, 25–47.
314. Tscherner, M.; Schwarzmüller, T.; Kuchler, K. Pathogenesis and Antifungal Drug Resistance of the Human Fungal Pathogen Candida glabrata. *Pharmaceuticals* **2011**, *4*, 169–186.
315. Calcagno, A.; Bignell, E.; Warn, P.; Jones, M.; Denning, D.; Muhlschlegel, F.; Rogers, T.; Haynes, K. C. glabrata STE12 is required for wild-type levels of virulence and nitrogen starvation induced filamentation. *Mol. Microbiol.* **2003**, *50*, 1309–1318.
316. Kanafani, Z. A.; Perfect, J. R. Resistance to antifungal agents: mechanisms and clinical impact. *Clin. Infect. Dis.* **2008**, *46*, 120–128.
317. Ferrari, S.; Sanguinetti, M.; De Bernardis, F.; Torelli, R.; Posteraro, B.; Vandeputte, P.; Sanglard, D. Loss of

mitochondrial functions associated with azole resistance in Candida glabrata results in enhanced virulence in mice. *Antimicrob. Agents Chemother.* **2011**, *55*, 1852–60.
318. Farahyar, S.; Zaini, F.; Kordbacheh, P.; Rezaie, S.; Falahati, M.; Safara, M. Expression of Efflux Pumps and Fatty Acid Activator One Genes in Azole Resistant Candida Glabrata Isolated From Immunocompromised Patients. *Acta Med. Iran.* **2015**, *54*, 458–464.
319. Kaur, R.; Castan, I.; Cormack, B. P. Functional Genomic Analysis of Fluconazole Susceptibility in the Pathogenic Yeast Candida glabrata : Roles of Calcium Signaling and Mitochondria. *Antimicrob. Agents Chemother.* **2004**, *48*, 1600–1613.
320. Miyazaki, T.; Yamauchi, S.; Inamine, T.; Nagayoshi, Y.; Saijo, T.; Izumikawa, K.; Seki, M.; Kakeya, H.; Yamamoto, Y.; Yanagihara, K.; Miyazaki, Y.; Kohno, S. Roles of calcineurin and Crz1 in antifungal susceptibility and virulence of Candida glabrata. *Antimicrob. Agents Chemother.* **2010**, *54*, 1639–43.
321. Salazar, S. B.; Wang, C.; Musterkotter, M.; Okamoto, M.; Takahashi-Nakaguchi, A.; Chibana, H.; Lopes, M. M.; Güldener, U.; Butler, G.; Mira, N. P. Comparative genomic and transcriptomic analyses unveil novel features of azole resistance and adaptation to the human host in Candida glabrata. *FEMS Yeast Res.* **2017**.
322. Morio, F.; Loge, C.; Besse, B.; Hennequin, C.; Le Pape, P. Screening for amino acid substitutions in the Candida albicans Erg11 protein of azole-susceptible and azole-resistant clinical isolates: new substitutions and a review of the literature. *Diagn. Microbiol. Infect. Dis.* **2010**, *66*, 373–384.
323. Marichal, P.; Koymans, L.; Willemsens, S.; Bellens, D.; Verhasselt, P.; Luyten, W.; Borgers, M.; Ramaekers, F. C.; Odds, F. C.; Bossche, H. V. Contribution of mutations in the cytochrome P450 14-alpha-demethylase (Erg11p, Cyp51p) to azole resistance in Candida albicans. *Microbiology* **1999**, *145*, 2701–2713.
324. Morio, F.; Pagniez, F.; Lacroix, C.; Miegeville, M.; Le Pape, P. Amino acid substitutions in the Candida albicans sterol D5,6-desaturase (Erg3p) confer azole resistance: characterization of two novel mutants with impaired virulence. *J. Antimicrob. Chemother.* **2012**, *67*, 2131–2138.
325. Sanglard, D.; Coste, A. T. Activity of isavuconazole and other azoles against Candida clinical isolates and yeast model systems with known azole resistance mechanisms. *Antimicrob. Agents Chemother.* **2016**, *60*.
326. Aramburu, J.; Rao, A.; Klee, C. B. Calcineurin: from structure to function. *Curr. Top. Cell. Regul.* **2000**, *36*, 237–95.
327. Onyewu, C.; Blankenship, J. R.; Del Poeta, M.; Heitman, J. Ergosterol biosynthesis inhibitors become fungicidal when combined with calcineurin inhibitors against Candida albicans, Candida glabrata, and Candida krusei. *Antimicrob. Agents Chemother.* **2003**, *47*, 956–64.
328. Cruz, M. C.; Goldstein, A. L.; Blankenship, J. R.; Del Poeta, M.; Davis, D.; Cardenas, M. E.; Perfect, J. R.; McCusker, J. H.; Heitman, J. Calcineurin is essential for survival during membrane stress in Candida albicans. *EMBO J.* **2002**, *21*, 546–59.
329. Berenguer, J.; Rodriguez-Tudela, J.; Richard, C.; Alvarez, M.; Sanz, M.; Gaztelurrutia, L.; Ayats, J.; Martinez-Suarez, J. Deep infections caused by Scedosporium prolificans. A report on 16 cases in Spain and a review of the literature. Scedosporium prolificans Spanish Study Group. *Med.* **1997**, *76*, 256–65.
330. Boutati, E.; Anaissie, E. Fusarium, a significant emerging pathogen in patients with hematologic malignancy: ten years' experience at a cancer center and implications for management. *Blood* **1997**, *90*, 999–1008.
331. Tritz, D.; Woods, G. Fatal disseminated infection with Aspergillus terreus in immunocompromised hosts. *Clin Infect Dis* **1993**, *16*, 118–22.
332. Rex, J. H. J.; Walsh, T. J.; Sobel, J. D. J.; Filler, S. G.; Pappas, P. G.; Dismukes, W. E.; Edwards, J. E. Practice guidelines for the treatment of candidiasis. *Clin Infect Dis* **2000**, *30*, 662–78.
333. Nolte, F.; Parkinson, T.; Falconer, D.; et al Isolation and characterization of fluconazole-and amphotericin B-resistant Candida albicans from blood of two patients with leukemia. *Antimicrob Agents Chemother* **1997**, *41*, 196–99.
334. Powderly, W.; Kobayashi, G.; Herzig, G.; Medoff, G. Amphotericin B-resistant yeast infection in severely immunocompromised patients. *Am J Med* **1988**, 826–32.
335. Hamilton-Miller JMT. Chemistry and biology of the polyene macrolide antibiotics. *Bacteriol Rev* **1973**, *37*, 166–96.
336. Vanden Bossche, H.; Marichal, H.; Odds, F. Molecular mechanisms of drug resistance in fungi. *Trends Microbiol* **1994**, *2*, 393–400.
337. Kelly, S. L.; Lamb, D. C.; Kelly, D. E.; Manning, N. J.; Loeffler, J.; Hebart, H.; Schumacher, U.; Einsele, H. Resistance to fluconazole and cross-resistance to amphotericin B in Candida albicans from AIDS patients caused by defective sterol delta 5,6-desaturation. *FEBS Lett.* **1997**, *400*, 80–82.
338. Pinjon, E.; Moran, G. P.; Jackson, C. J.; Kelly, S. L.; Sanglard, D.; Coleman, D. C.; Sullivan, D. J. Molecular mechanisms of itraconazole resistance in Candida dubliniensis. *Antimicrob. Agents Chemother.* **2003**, *47*, 2424–2437.
339. Martel, C. M.; Parker, J. E.; Bader, O.; Weig, M.; Gross, U.; Warrilow, A. G. S.; Rolley, N.; Kelly, D. E.; Kelly, S. L.

Identification and characterization of four azole-resistant erg3 mutants of Candida albicans. *Antimicrob. Agents Chemother.* **2010**, *54*, 4527–4533.
340. Martel, C. M.; Parker, J. E.; Bader, O.; Weig, M.; Gross, U.; Warrilow, A. G. S.; Kelly, D. E.; Kelly, S. L. A clinical isolate of Candida albicans with mutations in ERG11 (encoding sterol 14alpha-demethylase) and ERG5 (encoding C22 desaturase) is cross resistant to azoles and amphotericin B. *Antimicrob. Agents Chemother.* **2010**, *54*, 3578–83.
341. Hull, C. M.; Bader, O.; Parker, J. E.; Weig, M.; Gross, U.; Warrilow, A. G. S.; Kelly, D. E.; Kelly, S. L. Two clinical isolates of Candida glabrata exhibiting reduced sensitivity to amphotericin B both harbor mutations in ERG2. *Antimicrob. Agents Chemother.* **2012**, *56*, 6417–6421.
342. Jensen, R. H.; Astvad, K. M. T.; Silva, L. V.; Sanglard, D.; Jørgensen, R.; Nielsen, K. F.; Mathiasen, E. G.; Doroudian, G.; Perlin, D. S.; Arendrup, M. C. Stepwise emergence of azole, echinocandin and amphotericin B multidrug resistance in vivo in Candida albicans orchestrated by multiple genetic alterations. *J. Antimicrob. Chemother.* **2015**, *70*.
343. Castanheira, M.; Messer, S. A.; Rhomberg, P. R.; Pfaller, M. A. Antifungal susceptibility patterns of a global collection of fungal isolates: results of the SENTRY Antifungal Surveillance Program (2013). *Diagn. Microbiol. Infect. Dis.* **2016**, *85*, 200–204.
344. Beyda, N. D.; John, J.; Kilic, A.; Alam, M. J.; Lasco, T. M.; Garey, K. W. FKS mutant Candida glabrata: Risk factors and outcomes in patients with candidemia. *Clin. Infect. Dis.* **2014**, *59*.
345. Posteraro, B.; Vella, A.; De Carolis, E.; Sanguinetti, M. Molecular Detection of Resistance to Echinocandins. In *Human Fungal Pathogen Identification: Methods and Protocols*; Lion, T., Ed.; Springer Science: New York, 2017; Vol. 1508, pp. 323–337.
346. Beyda, N. D.; Lewis, R. E.; Garey, K. W. Echinocandin resistance in Candida species: mechanisms of reduced susceptibility and therapeutic approaches. *Ann. Pharmacother.* **2012**, *46*, 1086–1096.
347. Walker, L. A.; Gow, N. A.; Munro, C. A. Fungal echinocandin resistance. *Fungal Genet. Biol.* **2010**, *47*, 117–126.
348. Barchiesi, F.; Spreghini, E.; Tomassetti, S.; Della Vittoria, A.; Arzeni, D.; Manso, E.; Scalise, G. Effects of caspofungin against Candida guilliermondii and Candida parapsilosis. *Antimicrob. Agents Chemother.* **2006**, *50*, 2719–27.
349. Vai, M.; Orlandi, I.; Cavadini, P.; Alberghina, L.; Popolo, L. Candida albicans homologue of GGP1/GAS1 gene is functional in Saccharomyces cerevisiae and contains the determinants for glycosylphosphatidylinositol attachment. *Yeast* **1996**, *12*, 361–8.
350. Healey, K. R.; Nagasaki, Y.; Zimmerman, M.; Kordalewska, M.; Park, S.; Zhao, Y.; Perlin, D. S. The Gastrointestinal Tract Is a Major Source of Echinocandin Drug Resistance in a Murine Model of Candida glabrata Colonization and Systemic Dissemination. *Antimicrob. Agents Chemother.* **2017**, *61*.
351. Stevens, D. A.; White, T. C.; Perlin, D. S.; Selitrennikoff, C. P. Studies of the paradoxical effect of caspofungin at high drug concentrations. *Diagnostic Microbiol. Infect. Dis.* **2005**, *51*, 173–178.
352. Chamilos, G.; Lewis, R. E.; Albert, N.; Kontoyiannis, D. P. Paradoxical Effect of Echinocandins across Candida Species In Vitro: Evidence for Echinocandin-Specific and Candida Species-Related Differences. *Antimicrob Agents Chemother.* **2007**, *51*, 2257–2259.
353. Balashov, S. V.; Park, S.; Perlin, D. S. Assessing resistance to the echinocandin antifun- gal drug caspofungin in Candida albicans by profiling mutations in FKS1. *Antimicrob. Agents Chemother.* **2006**, 50, 2058–2063.
354. Zhao, Y.; Prideaux, B.; Nagasaki, Y.; Lee, M. H.; Chen, P.-Y.; Blanc, L.; Ho, H.; Clancy, C. J.; Nguyen, M. H.; Dartois, V.; Perlin, D. S. Unraveling Drug Penetration of Echinocandin Antifungals at the Site of Infection in an Intra-abdominal Abscess Model. *Antimicrob. Agents Chemother.* **2017**, *61*, e01009-17.
355. Lackner, M.; Tscherner, M.; Schaller, M.; Kuchler, K.; Mair, C.; Sartori, B.; Istel, F.; Arendrup, M. C.; Lass-Flörl, C. Positions and Numbers of *FKS* Mutations in Candida albicans Selectively Influence *In Vitro* and *In Vivo* Susceptibilities to Echinocandin Treatment. *Antimicrob. Agents Chemother.* **2014**, *58*, 3626–3635.
356. Arendrup, M. C.; Patterson, T. F. Multidrug-Resistant Candida: Epidemiology, Molecular Mechanisms, and Treatment. *J. Infect. Dis.* **2017**, *216*, 216–445.
357. Lackner, M.; Tscherner, M.; Schaller, M.; Kuchler, K.; Mair, C.; Sartori, B.; Istel, F.; Arendrup, M. C.; Lass-Fl??rl, C. Positions and numbers of FKS mutations in Candida albicans selectively influence In Vitro and In Vivo susceptibilities to echinocandin treatment. *Antimicrob. Agents Chemother.* **2014**, *58*.
358. Dixon, D. M.; Walsh, T. J. Antifungal Agents. In *Medical Microbiology*.; S, B., Ed.; Galveston (TX): University of Texas Medical Branch at Galveston, 1996.
359. Erbs, P.; Exinger, F.; Jund, R. Characterization of the Saccharomyces cerevisiae FCY1 gene encoding cytosine deaminase and its homologue FCA1 of Candida albicans. *Curr. Genet.* **1997**, *31*, 1–6.
360. Charlier, C.; El Sissy, C.; Bachelier-Bassi, S.; Scemla, A.; Quesne, G.; Sitterlé, E.; Legendre, C.; Lortholary, O.; Bougnoux, M.-E. Acquired Flucytosine Resistance during Combination Therapy with Caspofungin and Flucytosine for Candida glabrata Cystitis. *Antimicrob. Agents Chemother.* **2015**, *60*, 662–5.

361. Biswas, C.; Chen, S. C.; Halliday, C.; Kennedy, K.; Playford, G.; Marriott, D. J.; Slavin, M. A.; Sorrell, T. C. Identification of genetic markers of resistance to echinocandins, azoles and 5-fluorocytosine in Candida glabrata by next generation sequencing: a feasibility study. *Clin. Microbiol. Infect.* **2017**.
362. Costa, C.; Ponte, A.; Pais, P.; Santos, R.; Cavalheiro, M.; Yaguchi, T.; Chibana, H.; Teixeira, M. C. New Mechanisms of Flucytosine Resistance in C. glabrata Unveiled by a Chemogenomics Analysis in S. cerevisiae. *PLoS One* **2015**, *10*, e0135110.
363. Dixon, D. M.; Walsh, T. J. *Antifungal Agents*; University of Texas Medical Branch at Galveston, 1996.
364. Odds, F. C. In *Candida albicans* , resistance to flucytosine and terbinafine is linked to *MAT* locus homozygosity and multilocus sequence typing clade 1. *FEMS Yeast Res.* **2009**, *9*, 1091–1101.
365. Vincent, B. M.; Lancaster, A. K.; Scherz-Shouval, R.; Whitesell, L.; Lindquist, S. Fitness Trade-offs Restrict the Evolution of Resistance to Amphotericin B. *PLoS Biol.* **2013**, *11*.
366. Ben-Ami, R.; Kontoyiannis, D. P. Resistance to echinocandins comes at a cost. *Virulence* **2012**, *3*, 95–97.
367. Wang, E.; Al., E. The ever-evolving landscape of candidaemia in patients with acute leukaemia: non-susceptibility to caspofungin and multidrug resistance are associated with increased mortality. *J Antimicrob Chemother* **2015**.
368. Cleveland, A. A.; Al., E. Declining incidence of candidemia and the shifting epidemiology of Candida resistance in two US metropolitan areas, 2008-2013: results from population-based surveillance. *PLoS One* **2015**, *10*, e0120452.
369. Razzaghi-Abyaneh, M.; Al., E. Species distribution and antifungal susceptibility of Candida spp. isolated from superficial candidiasis in outpatients in Iran. *J Mycol Med* **2014**, *24*, e43–50.
370. Sanguinetti, M.; Posteraro, B.; Lass-Florl, C. Antifungal drug resistance among Candida species: mechanisms and clinical impact. *Mycoses 58 Suppl* **2015**, *2*, 2–13.
371. Lortholary, O.; Desnos-Ollivier, M.; Sitbon, K.; Fontanet, A.; Bretagne, S.; Dromer, F.; French Mycosis Study Group Recent Exposure to Caspofungin or Fluconazole Influences the Epidemiology of Candidemia: a Prospective Multicenter Study Involving 2,441 Patients. *Antimicrob. Agents Chemother.* **2011**, *55*, 532–538.
372. Pappas, P. G.; Kauffman, C. A.; Andes, D. R.; Clancy, C. J.; Marr, K. A.; Ostrosky-Zeichner, L.; Reboli, A. C.; Schuster, M. G.; Vazquez, J. A.; Walsh, T. J.; Zaoutis, T. E.; Sobel, J. D. Clinical Practice Guideline for the Management of Candidiasis: 2016 Update by the Infectious Diseases Society of America. *Clin. Infect. Dis.* **2015**, *62*, e1–e50.
373. M., A.; Sulim, C. S.; Holm, A.; Nielsen, L.; Nielsen, S. D.; Knudsen, J. D.; Drenck, N. E.; Christensen, J. J.; Johansen, H. K. Diagnostic issues, clinical characteristics, and outcomes for patients with fungemia. *J Clin.Microbiol* **2011**, *49*, 3300–3308.
374. Cornely, O. A.; Bassetti, M.; Calandra, T.; Garbino, J.; Kullberg, B. J.; Lortholary, O.; W., M.; Akova, M.; Arendrup, M. C.; Arikan-Akdagli, S.; Al., E. ESCMID* guideline for the diagnosis and management of Candida diseases 2012: non-neutropenic adult patients. *Clin. Microbiol. Infect. 18 (Suppl. 7)* **2012**, 19–37.
375. Healey, K. R.; Zhao, Y.; Perez, W. B.; Lockhart, S. R.; Sobel, J. D.; Farmakiotis, D.; Kontoyiannis, D. P.; Sanglard, D.; Taj-Aldeen, S. J.; Alexander, B. D.; Jimenez-Ortigosa, C.; Shor, E.; Perlin, D. S. Prevalent mutator genotype identified in fungal pathogen Candida glabrata promotes multi-drug resistance. *Nat Commun* **2016**, *7*, 11128.
376. Dellière, S.; Healey, K.; Gits-Muselli, M.; Carrara, B.; Barbaro, A.; Guigue, N.; Lecefel, C.; Touratier, S.; Desnos-Ollivier, M.; Perlin, D. S.; Bretagne, S.; Alanio, A. Fluconazole and Echinocandin Resistance of Candida glabrata Correlates Better with Antifungal Drug Exposure Rather than with MSH2 Mutator Genotype in a French Cohort of Patients Harboring Low Rates of Resistance. *Front. Microbiol.* **2016**, *7*, 2038.
377. Vallabhaneni, S.; Cleveland, A. A.; Farley, M. M.; Harrison, L. H.; Schaffner, W.; Beldavs, Z. G.; Derado, G.; Pham, C. D.; Lockhart, S. R.; Smith, R. M. Epidemiology and Risk Factors for Echinocandin Nonsusceptible Candida glabrata Bloodstream Infections: Data From a Large Multisite Population-Based Candidemia Surveillance Program, 2008-2014. *Open forum Infect. Dis.* **2015**, *2*, ofv163.
378. Jensen, R. H.; Johansen, H. K.; Søes, L. M.; Lemming, L. E.; Rosenvinge, F. S.; Nielsen, L.; Olesen, B.; Kristensen, L.; Dzajic, E.; Astvad, K. M. T.; Arendrup, M. C. Posttreatment antifungal resistance among colonizing Candida isolates in candidemia patients: Results from a systematic multicenter study. *Antimicrob. Agents Chemother.* **2016**, *60*.
379. Shields, R. K.; Nguyen, M. H.; Press, E. G.; Clancya, C. J. Abdominal candidiasis is a hidden reservoir of echinocandin resistance. *Antimicrob. Agents Chemother.* **2014**, *58*.
380. Farmakiotis, D.; Tarrand, J. J.; Kontoyiannis, D. P. Drug-resistant Candida glabrata infection in cancer patients. *Emerg Infect Dis* **2014**, *20*, 1833–40.
381. Mohamadi, J.; Motaghi, M.; Panahi, J.; Havasian, M. R.; Delpisheh, A.; Azizian, M.; Pakzad, I. Anti-fungal resistance in candida isolated from oral and diaper rash candidiasis in neonates. *Bioinformation* **2014**, *10*, 667–70.
382. Nieto, M. C.; Tellería, O.; Cisterna, R. Sentinel surveillance of invasive candidiasis in Spain: epidemiology and antifungal susceptibility. *Diagn. Microbiol. Infect. Dis.* **2015**, *81*, 34–40.
383. Pfaller, M. A.; Rhomberg, P. R.; Messer, S. A.; Jones, R. N.; Castanheira, M. Isavuconazole, micafungin, and 8 comparator antifungal agents' susceptibility profiles for common and uncommon opportunistic fungi collected in 2013. *Diagn. Microbiol. Infect. Dis.* **2015**, *82*, 303–13.

384. Schmalreck, A. F.; Willinger, B.; Haase, G.; Blum, G.; Lass-Flörl, C.; Fegeler, W.; Becker, K.; Antifungal Susceptibility Testing-AFST Study Group Species and susceptibility distribution of 1062 clinical yeast isolates to azoles, echinocandins, flucytosine and amphotericin B from a multi-centre study. *Mycoses* **2012**, *55*, e124-37.
385. Shi, X.-Y.; Yang, Y.-P.; Zhang, Y.; Li, W.; Wang, J.-D.; Huang, W.-M.; Fan, Y.-M. Molecular identification and antifungal susceptibility of 186 Candida isolates from vulvovaginal candidiasis in southern China. *J. Med. Microbiol.* **2015**, *64*, 390–3.
386. Zarei Mahmoudabadi, A.; Rezaei-Matehkolaei, A.; Ghanavati, F. The Susceptibility Patterns of Candida Species Isolated From Urine Samples to Posaconazole and Caspofungin. *Jundishapur J. Microbiol.* **2015**, *8*.
387. Posteraro, B.; Spanu, T.; Fiori, B.; De Maio, F.; De Carolis, E.; Giaquinto, A.; Prete, V.; De Angelis, G.; Torelli, R.; D'Inzeo, T.; Vella, A.; De Luca, A.; Tumbarello, M.; Ricciardi, W.; Sanguinetti, M. Antifungal susceptibility profiles of bloodstream yeast isolates by sensititre yeastone over nine years at a large Italian teaching hospital. *Antimicrob. Agents Chemother.* **2015**, *59*, 3944–3955.
388. Czaika, V.; Nenoff, P.; Glöckner, A.; Becker, K.; Fegeler, W.; Schmalreck, A. F. Detection of azole susceptibility patterns in clinical yeast strains isolated from 1998 to 2008. *New Microbiol.* **2014**, *37*, 465–94.
389. Pfaller, M. A.; Messer, S. A.; Boyken, L.; Tendolkar, S.; Hollis, R. J.; Diekema, D. J. Geographic variation in the susceptibilities of invasive isolates of Candida glabrata to seven systemically active antifungal agents: a global assessment from the ARTEMIS Antifungal Surveillance Program conducted in 2001 and 2002. *J. Clin. Microbiol.* **2004**, *42*, 3142–6.
390. Gopinathan, S.; Janagond, A. B.; Agatha, D.; P R, T. Detection of FUR1 Gene in 5-Flucytosine Resistant Candida Isolates in Vaginal Candidiasis Patients. *J. Clin. Diagn. Res.* **2013**, *7*, 2452–5.
391. Haddadi, P.; Zareifar, S.; Badiee, P.; Alborzi, A.; Mokhtari, M.; Zomorodian, K.; Pakshir, K.; Jafarian, H. Yeast Colonization and Drug Susceptibility Pattern in the Pediatric Patients With Neutropenia. *Jundishapur J. Microbiol.* **1970**, *7*, 1–6.
392. Andrade-Silva, L.; Ferreira-Paim, K.; Mora, D. J.; Silva, P. R. Da; Andrade, A. A.; Araujo, N. E.; Pedrosa, A. L.; Silva-Vergara, M. L. Susceptibility profile of clinical and environmental isolates of *Cryptococcus neoformans* and *Cryptococcus gattii* in Uberaba, Minas Gerais, Brazil. *Med. Mycol.* **2013**, *51*, 635–640.
393. Oxman, D. A.; Chow, J. K.; Frendl, G.; Hadley, S.; Hershkovitz, S.; Ireland, P.; McDermott, L. A.; Tsai, K.; Marty, F. M.; Kontoyiannis, D. P.; Golan, Y. Candidaemia associated with decreased in vitro fluconazole susceptibility: is Candida speciation predictive of the susceptibility pattern? *J. Antimicrob. Chemother.* **2010**, *65*, 1460–5.
394. Pfaller, M. A.; Messer, S. A.; Jones, R. N.; Castanheira, M. Antifungal susceptibilities of Candida, Cryptococcus neoformans and Aspergillus fumigatus from the Asia and Western Pacific region: data from the SENTRY antifungal surveillance program (2010–2012). *J. Antibiot. (Tokyo).* **2015**, *68*, 556–561.
395. Lee, C.-H.; Chang, T.-Y.; Liu, J.-W.; Chen, F.-J.; Chien, C.-C.; Tang, Y.-F.; Lu, C.-H. Correlation of anti-fungal susceptibility with clinical outcomes in patients with cryptococcal meningitis. *BMC Infect. Dis.* **2012**, *12*, 361.
396. Matos, C. S.; de Souza Andrade, A.; Oliveira, N. S.; Barros, T. F. Microbiological characteristics of clinical isolates of Cryptococcus spp. in Bahia, Brazil: molecular types and antifungal susceptibilities. *Eur. J. Clin. Microbiol. Infect. Dis.* **2012**, *31*, 1647–52.
397. Asadzadeh, M.; Al-Sweih, N. A.; Ahmad, S.; Khan, Z. U. Antifungal susceptibility of clinical Candida parapsilosis isolates in Kuwait. *Mycoses* **2008**, *51*, 318–23.
398. Hegazi, M.; Abdelkader, A.; Zaki, M.; El-Deek, B. Characteristics and risk factors of candidemia in pediatric intensive care unit of a tertiary care children's hospital in Egypt. *J. Infect. Dev. Ctries.* **2014**, *8*, 624–34.
399. Zaoutis, T. E.; Foraker, E.; McGowan, K. L.; Mortensen, J.; Campos, J.; Walsh, T. J.; Klein, J. D. Antifungal susceptibility of Candida spp. isolated from pediatric patients: a survey of 4 children's hospitals. *Diagn. Microbiol. Infect. Dis.* **2005**, *52*, 295–8.
400. Krogh-Madsen, M.; Arendrup, M. C. M. C.; Heslet, L.; Knudsen, J. D. J. D. Amphotericin B and caspofungin resistance in Candida glabrata isolates recovered from a critically ill patient. *Clin. Infect. Dis.* **2006**, *42*, 938–944.
401. Eddouzi, J.; Parker, J. E.; Vale-Silva, L. A.; Coste, A.; Ischer, F.; Kelly, S.; Manai, M.; Sanglard, D. Molecular mechanisms of drug resistance in clinical Candida species isolated from Tunisian hospitals. *Antimicrob. Agents Chemother.* **2013**, 3182–3193.
402. Forastiero, A.; Mesa-Arango, A. C.; Alastruey-Izquierdo, A.; Alcazar-Fuoli, L.; Bernal-Martinez, L.; Pelaez, T.; Lopez, J. F.; Grimalt, J. O.; Gomez- Lopez, A.; Cuesta, I.; Zaragoza, O.; Mellado, E. Candida tropicalis antifungal cross-resistance is related to different azole target (Erg11p) modifications. *Antimicrob. Agents Chemother.* **2013**, *57*, 4769–4781.
403. Costa, C.; Pais, P.; Teixeira, M. C. Multidrug resistance in pathogenic yeasts : emphasis on the role of ABC and MFS multidrug transporters. In *The Battle Against Microbial Pathogens: Basic Science, Technological Advances and Educational Programs (A. Méndez-Vilas, Ed.)*; 2015; pp. 947–954.
404. Sanglard, D.; Ischer, F.; Bille, J. Role of ATP-binding-cassette transporter genes in high-frequency acquisition of resistance to azole antifungals in Candida glabrata. *Antimicrob. Agents Chemother.* **2001**, *45*, 1174–1183.

405. Torelli, R.; Posteraro, B.; Ferrari, S.; La Sorda, M.; Fadda, G.; Sanglard, D.; Sanguinetti, M. The ATP-binding cassette transporter-encoding gene CgSNQ2 is contributing to the CgPDR1-dependent azole resistance of Candida glabrata. *Mol. Microbiol.* **2008**, *68*, 186–201.
406. Orta-Zavalza, E.; Guerrero-Serrano, G.; Gutiérrez-Escobedo, G.; Cañas-Villamar, I.; Juárez-Cepeda, J.; Castaño, I.; De Las Peñas, A. Local silencing controls the oxidative stress response and the multidrug resistance in Candida glabrata. *Mol. Microbiol.* **2013**, *88*, 1135–48.
407. Costa, C.; Pires, C.; Cabrito, T. R.; Renaudin, A.; Ohno, M.; Chibana, H.; Sá-Correia, I.; Teixeira, M. C. Candida glabrata drug:H+ antiporter CgQdr2 confers imidazole drug resistance, being activated by transcription factor CgPdr1. *Antimicrob. Agents Chemother.* **2013**, *57*, 3159–67.
408. Costa, C.; Al., E. Candida glabrata drug:H+ antiporter CgTpo3 (ORF CAGL0I10384g): role in azole drug resistance and polyamine homeostasis. *J Antimicrob Chemother* **2014**, *69*, 1767–76.
409. Costa, C.; Henriques, A.; Pires, C.; Nunes, J.; Ohno, M.; Chibana, H.; Sá-Correia, I.; Teixeira, M. C. The dual role of candida glabrata drug:H+ antiporter CgAqr1 (ORF CAGL0J09944g) in antifungal drug and acetic acid resistance. *Front. Microbiol.* **2013**, *4*, 170.
410. Hull, C. M.; Parker, J. E.; Bader, O.; Weig, M.; Gross, U.; Warrilow, A. G. S.; Kelly, D. E.; Kelly, S. L. Facultative sterol uptake in an ergosterol-deficient clinical isolate of candida glabrata harboring a missense mutation in ERG11 and exhibiting cross-resistance to azoles and amphotericin B. *Antimicrob. Agents Chemother.* **2012**, *56*, 4223–4232.
411. Brun, S.; Bergès, T.; Poupard, P.; Vauzelle-Moreau, C.; Renier, G.; Chabasse, D.; Bouchara, J.-P. Mechanisms of azole resistance in petite mutants of Candida glabrata. *Antimicrob. Agents Chemother.* **2004**, *48*, 1788–1796.
412. Ferrari, S.; Ischer, F.; Calabrese, D.; Posteraro, B.; Sanguinetti, M.; Fadda, G.; Rohde, B.; Bauser, C.; Bader, O.; Sanglard, D. Gain of function mutations in CgPDR1 of Candida glabrata not only mediate antifungal resistance but also enhance virulence. *PLoS Pathog.* **2009**, *5*, e1000268.
413. Mansfield, B. E.; Oltean, H. N.; Oliver, B. G.; Hoot, S. J.; Leyde, S. E.; Hedstrom, L.; White, T. C.; BE, M. Azole drugs are imported by facilitated diffusion in Candida albicans and other pathogenic fungi. *PLoS Pathog.* **2010**, *6*, e1001126.
414. Miranda, I. M.; Silva, R. M.; Estevinho, M.; Pina-vaz, C. Environmental azole fungicide, prochloraz, can induce cross-resistance to medical triazoles in Candida glabrata. *FEMS Yeast Res.* **2014**, 1–5.
415. Rodrigues, M. E.; Henriques, M.; Silva, S. Disinfectants to Fight Oral Candida Biofilms. In *Advances in experimental medicine and biology*, 2016; Vol. 931, pp. 83–93.
416. Ramage, G.; Wickes, B. L.; Lopez-Ribot, J. L. Biofilms of Candida albicans and their associated resistance to antifungal agents. *Am. Clin. Lab.* **2001**, *20*, 42–4.
417. Bandara, H. M. H. N.; Matsubara, V. H.; Samaranayake, L. P. Future therapies targeted towards eliminating Candida biofilms and associated infections. *Expert Rev. Anti. Infect. Ther.* **2017**, *15*, 299–318.
418. Freire, F.; Ferraresi, C.; Jorge, A. O. C.; Hamblin, M. R. Photodynamic therapy of oral Candida infection in a mouse model. *J. Photochem. Photobiol. B Biol.* **2016**, *159*, 161–168.
419. de Souza, S. C.; Junqueira, J. C.; Balducci, I.; Koga-Ito, C. Y.; Munin, E.; Jorge, A. O. C. Photosensitization of different Candida species by low power laser light. *J. Photochem. Photobiol. B Biol.* **2006**, *83*, 34–38.
420. Souza, R. C.; Junqueira, J. C.; Rossoni, R. D.; Pereira, C. A.; Munin, E.; Jorge, A. O. C. Comparison of the photodynamic fungicidal efficacy of methylene blue, toluidine blue, malachite green and low-power laser irradiation alone against Candida albicans. *Lasers Med. Sci.* **2010**, *25*, 385–389.
421. Giroldo, L. M.; Felipe, M. P.; de Oliveira, M. A.; Munin, E.; Alves, L. P.; Costa, M. S. Photodynamic antimicrobial chemotherapy (PACT) with methylene blue increases membrane permeability in Candida albicans. *Lasers Med. Sci.* **2009**, *24*, 109–112.
422. Dovigo, L. N.; Pavarina, A. C.; de Oliveira Mima, E. G.; Giampaolo, E. T.; Vergani, C. E.; Bagnato, V. S. Fungicidal effect of photodynamic therapy against fluconazole-resistant Candida albicans and Candida glabrata. *Mycoses* **2011**, *54*, 123–130.
423. Mima, E. G. de O.; Pavarina, A. C.; Dovigo, L. N.; Vergani, C. E.; Costa, C. A. de S.; Kurachi, C.; Bagnato, V. S. Susceptibility of Candida albicans to photodynamic therapy in a murine model of oral candidosis. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* **2010**, *109*, 392–401.
424. Dahanukar, S. A.; Kulkarni, R. A.; Rege, N. N.; A, Nd. S.; Kulkarni, R. A.; Rege, N. N. Pharmacology of medicinal plants and natural products. *Indian J. Pharmacol.* **2000**, *32*, S81–S118.
425. Cowan, M. Plant products as antimicrobial agents. *Clin. Microbiol. Rev.* **1999**, *12*, 564–82.
426. Pozzatti, P.; Scheid, L.; Spader, T.; Atayde, M.; Santurio, J.; Alves, S. In vitro activity of essential oils extracted from plants used as spices against fluconazole-resistant and fluconazole-susceptible Candida spp. Canadian. *Can. J. Microbiol.* **2008**, *54*, 950–956.
427. Martins, N.; Ferreira, I. C. F. R.; Henriques, M.; Silva, S. In vitro anti-Candida activity of Glycyrrhiza glabra L. *Ind. Crops Prod.* **2016**, *83*, 81–85.
428. Martins, N.; Ferreira, I. C. F. R.; Barros, L.; Carvalho, A. M.; Henriques, M.; Silva, S. Plants used in folk medicine:

The potential of their hydromethanolic extracts against Candida species. *Ind. Crops Prod.* **2015**, *66*, 62–67.
429. Prabhakar, K.; Kumar, L. S.; Rajendran, S.; Chandrasekaran, M.; Bhaskar, K.; Sajit Khan, A. K. Antifungal Activity of Plant Extracts against Candida Species from Oral Lesions. *Indian J. Pharm. Sci.* **2008**, *70*, 801–803.
430. Nazzaro, F.; Fratianni, F.; Coppola, R.; Feo, V. De Essential Oils and Antifungal Activity. *Pharmaceuticals* **2017**, *10*, 86.
431. Eteraf-Oskouei, T.; Najafi, M. Traditional and Modern Uses of Natural Honey in Human Diseases: A Review. *Iran. J. Basic Med. Sci.* **2013**, *16*, 731–742.
432. Theunissen, F.; Grobler, S.; Gedalia, I. The antifungal action of three South African honeys on Candida albicans. *Apidologie* **2001**, *32*, 371–379.
433. Irish, J.; Carter, D.; Shokohi, T.; Blair, S. Honey has an antifungal effect against Candida species. *Med. Mycol.* **2006**, *44*, 289–291.
434. Küçük, M.; Kolaylı, S.; Karaoğlu, Ş.; Ulusoy, E.; Baltacı, C.; Candan, F. Biological activities and chemical composition of three honeys of different types from Anatolia. *Food Chem.* **2007**, *100*, 526–534.
435. Boukraa, L.; Benbarek, H.; Moussa, A. Synergistic action of starch and honey against Candida albicans in correlation with diastase number. *Brazilian J. Microbiol.* **2008**, *39*, 40–43.
436. Koc, A. N.; Silici, S.; Ercal, B. D.; Kasap, F.; Hörmet-Öz, H. T.; Mavus-Buldu, H. Antifungal Activity of Turkish Honey against Candida spp. and Trichosporon spp: an in vitro evaluation. *Med. Mycol.* **2009**, *47*, 707–712.
437. Tobaldini-Valerio, F. K.; Bonfim-Mendonça, P. S.; Rosseto, H. C.; Bruschi, M. L.; Henriques, M.; Negri, M.; Silva, S.; Svidzinski, T. I. Propolis: a potential natural product to fight *Candida* species infections. *Future Microbiol.* **2016**, *11*, 1035–1046.
438. Quadri, M.; Huraib, S. Manuka Honey for Central Vein Catheter Exit Site Care. *Semin. Dial.* **1999**, *12*, 396–399.
439. Johnson, D. W.; van Eps, C.; Mudge, D. W.; Wiggins, K. J.; Armstrong, K.; Hawley, C. M.; Campbell, S. B.; Isbel, N. M.; Nimmo, G. R.; Gibbs, H. Randomized, Controlled Trial of Topical Exit-Site Application of Honey (Medihoney) versus Mupirocin for the Prevention of Catheter-Associated Infections in Hemodialysis Patients. *J. Am. Soc. Nephrol.* **2005**, *16*, 1456–1462.
440. Miyazima, T.; Ishikawa, K.; Mayer, M.; Saad, S.; Nakamae, A. Cheese supplemented with probiotics reduced the Candida levels in denture wearers - RCT. *Oral Dis.* **2017**, *23*, 919–925.
441. Oliveira, V. M. C.; Santos, S. S. F.; Silva, C. R. G.; Jorge, A. O. C.; Le??o, M. V. P. Lactobacillus is able to alter the virulence and the sensitivity profile of Candida albicans. *J. Appl. Microbiol.* **2016**, *121*, 1737–1744.
442. Wagner, R. D.; Pierson, C.; Warner, T.; Dohnalek, M.; Farmer, J.; Roberts, L.; Hilty, M.; Balish, E. Biotherapeutic Effects of Probiotic Bacteria on Candidiasis in Immunodeficient Mice. *Infect. Immun.* **1997**, *65*, 4165–4172.
443. Houte, J. Van; Gibbons, R. J.; Pulkkinen, A. J. Ecology of Human Oral Lactobacilli. *Infect. Immun.* **1972**, *6*, 723–729.
444. Haukioja, A. Probiotics and Oral Health. *Eur. J. Dent.* **2010**, *4*, 348–355.
445. Krasse, P.; Carlsson, B.; Dahl, C.; Paulsson, A.; Nilsson, A.; Sinkiewicz, G. Decreased gum bleeding and reduced gingivitis by the probiotic Lactobacillus reuteri. *Swed. Dent. J.* **2006**, *30*, 55–60.
446. Vivekananda, M. R.; Vandana, K. L.; Bhat, K. G. Effect of the probiotic Lactobacilli reuteri (Prodentis) in the management of periodontal disease: a preliminary randomized clinical trial. *J. Oral Microbiol.* **2010**, *2*.
447. Cagetti, M. G.; Mastroberardino, S.; Milia, E.; Cocco, F.; Lingström, P.; Campus, G. The Use of Probiotic Strains in Caries Prevention: A Systematic Review. *Nutrients* **2013**, *5*, 2530–2550.
448. Ng, T. B.; Wright, A. Von; Ohshima, T.; Kojima, Y.; Seneviratne, C. J.; Maeda, N. Therapeutic Application of Synbiotics, a Fusion of Probiotics and Prebiotics, and Biogenics as a New Concept for Oral Candida Infections: A Mini Review. *Front. Microbiol* **2016**, *7*, 3389–10.
449. Demirel, G.; Celik, I. H.; Erdeve, O.; Saygan, S.; Dilmen, U.; Canpolat, F. E. Prophylactic Saccharomyces boulardii versus nystatin for the prevention of fungal colonization and invasive fungal infection in premature infants. *Eur. J. Pediatr.* **2013**, *172*, 1321–1326.
450. Kumar, S.; Singhi, S.; Chakrabarti, A.; Bansal, A.; Jayashree, M. Probiotic Use and Prevalence of Candidemia and Candiduria in a PICU. *Pediatr. Crit. Care Med.* **2013**, *14*, e409–e415.
451. Matsubara, V. H.; Bandara, H. M. H. N.; Mayer, M. P. A.; Samaranayake, L. P. Probiotics as Antifungals in Mucosal Candidiasis. *Clin. Infect. Dis.* **2016**, *62*, 1143–1153.
452. Ishikawa, K. H.; Mayer, M. P. A.; Miyazima, T. Y.; Matsubara, V. H.; Silva, E. G.; Paula, C. R.; Campos, T. T.; Nakamae, A. E. M. A Multispecies Probiotic Reduces Oral *Candida* Colonization in Denture Wearers. *J. Prosthodont.* **2015**, *24*, 194–199.
453. Jørgensen, M. R.; Keller, M. K.; Kragelund, C.; Twetman, S. Effect of Probiotic Bacteria on Oral Candida in Frail Elderly. *JDR Clin. Res. Suppl.* **2012**, *94*, 181S–186S.
454. Li, D.; Li, Q.; Liu, C.; Lin, M.; Li, X.; Xiao, X.; Zhu, Z.; Gong, Q.; Zhou, H. Efficacy and safety of probiotics in the treatment of Candida-associated stomatitis. *Mycoses* **2014**, *57*, 141–146.

455. Mendonça, F. H. B. P.; Santos, S. S. F. dos; Faria, I. da S. de; Gonçalves e Silva, C. R.; Jorge, A. O. C.; Leão, M. V. P. Effects of probiotic bacteria on Candida presence and IgA anti-Candida in the oral cavity of elderly. *Braz. Dent. J.* **2012**, *23*, 534–8.
456. Hatakka, K.; Ahola, A. J.; Yli-Knuuttila, H.; Richardson, M.; Poussa, T.; Meurman, J. H.; Korpela, R. Probiotics Reduce the Prevalence of Oral *Candida* in the Elderly—a Randomized Controlled Trial. *J. Dent. Res.* **2007**, *86*, 125–130.
457. Fuller, R. Probiotics in man and animals. *J. Appl. Bacteriol.* **1989**, *66*, 365–78.
458. Sanders, M. E. Probiotics: Definition, Sources, Selection, and Uses. *Clin. Infect. Dis.* **2008**, *46*, S58–S61.
459. Gourbeyre, P.; Denery, S.; Bodinier, M. Probiotics, prebiotics, and synbiotics: impact on the gut immune system and allergic reactions. *J. Leukoc. Biol.* **2011**, *89*, 685–695.
460. James, K. M.; MacDonald, K. W.; Chanyi, R. M.; Cadieux, P. A.; Burton, J. P. Inhibition of Candida albicans biofilm formation and modulation of gene expression by probiotic cells and supernatant. *J. Med. Microbiol.* **2016**, *65*, 328–336.
461. Larmas, M.; Mäkinen, K. K.; Scheinin, A. Turku sugar studies. III. An intermediate report on the effect of sucrose, fructose and xylitol diets on the numbers of salivary lactobacilli, candida and streptococci. *Acta Odontol. Scand.* **1974**, *32*, 423–33.
462. Larmas, M.; Mäkinen, K. K.; Scheinin, A. Turku sugar studies. VIII. Principal microbiological findings. *Acta Odontol. Scand.* **1976**, *34*, 285–328.
463. Pizzo, G.; Giuliana, G.; Milici, M. E.; Giangreco, R. Effect of dietary carbohydrates on the in vitro epithelial adhesion of Candida albicans, Candida tropicalis, and Candida krusei. *New Microbiol.* **2000**, *23*, 63–71.
464. Abu-Elteen, K. H. The influence of dietary carbohydrates on *in vitro* adherence of four *Candida* species to human buccal epithelial cells. *Microb. Ecol. Health Dis.* **2005**, *17*, 156–162.
465. de Souza, T. B.; de Oliveira Brito, K. M.; Silva, N. C.; Rocha, R. P.; de Sousa, G. F.; Duarte, L. P.; Coelho, L. F. L.; Dias, A. L. T.; Veloso, M. P.; Carvalho, D. T.; Dias, D. F. New Eugenol Glucoside-based Derivative Shows Fungistatic and Fungicidal Activity against Opportunistic *Candida glabrata*. *Chem. Biol. Drug Des.* **2015**, n/a-n/a.
466. Casanova, B. B.; Muniz, M. N.; Oliveira, T. de; Oliveira, L. F. de; Machado, M. M.; Fuentefria, A. M.; Gosmann, G.; Gnoatto, S. C. B. Synthesis and Biological Evaluation of Some Hydrazone Derivatives as Anti-inflammatory Agents. *Lett. Drug Des. Discov.* **2012**, *9*, 310–315.
467. Ferreira, B. D. S.; de Almeida, A. M.; Nascimento, T. C.; de Castro, P. P.; Silva, V. L.; Diniz, C. G.; Le Hyaric, M. Synthesis and biological evaluation of a new series of N-acyldiamines as potential antibacterial and antifungal agents. *Bioorg. Med. Chem. Lett.* **2014**, *24*, 4626–4629.
468. Abrão, P. H. O.; Pizi, R. B.; de Souza, T. B.; Silva, N. C.; Fregnan, A. M.; Silva, F. N.; Coelho, L. F. L.; Malaquias, L. C. C.; Dias, A. L. T.; Dias, D. F.; Veloso, M. P.; Carvalho, D. T. Synthesis and Biological Evaluation of New Eugenol Mannich Bases as Promising Antifungal Agents. *Chem. Biol. Drug Des.* **2015**, n/a-n/a.
469. Silva-Dias, A.; Miranda, I. M.; Branco, J.; Cobrado, L.; Monteiro-Soares, M.; Pina-Vaz, C.; Rodrigues, A. G. In vitro antifungal activity and in vivo antibiofilm activity of cerium nitrate against Candida species. *J. Antimicrob. Chemother.* **2014**, *70*, 1083–1093.
470. Joseph, M. R. P.; Al-Hakami, a. M.; Assiry, M. M.; Jamil, a. S.; Assiry, a. M.; Shaker, M. a.; Hamid, M. E. In vitro anti-yeast activity of chloramphenicol: A preliminary report. *J. Mycol. Médicale / J. Med. Mycol.* **2015**, *25*, 17–22.
471. Ning, Y.; Ling, J.; Wu, C. D. Synergistic effects of tea catechin epigallocatechin gallate and antimycotics against oral Candida species. *Arch. Oral Biol.* **2015**, *60*, 1565–1570.
472. Perera, J.; Weerasekera, M.; Kottegoda, N. Slow release anti-fungal skin formulations based on citric acid intercalated layered double hydroxides nanohybrids. *Chem. Cent. J.* **2015**, *9*, 27.
473. Silva, S.; Pires, P.; Monteiro, D. R.; Negri, M.; Gorup, L. F.; Camargo, E. R.; Barbosa, D. B.; Oliveira, R.; Williams, D. W.; Henriques, M.; Azeredo, J. The effect of silver nanoparticles and nystatin on mixed biofilms of Candida glabrata and Candida albicans on acrylic. *Med. Mycol.* **2013**, *51*, 178–84.
474. Nirma, C.; Eparvier, V.; Stien, D. Antifungal Agents from *Pseudallescheria boydii* SNB-CN73 Isolated from a *Nasutitermes* sp. Termite. *J. Nat. Prod.* **2013**, *76*, 988–991.
475. Nirma, C.; Eparvier, V.; Stien, D. Antibacterial ilicicolinic acids C and D and ilicicolinal from neonectria discophora SNB-CN63 isolated from a termite nest. *J. Nat. Prod.* **2015**, *78*, 159–162.
476. Casella, T. M.; Eparvier, V.; Mandavid, H.; Bendelac, A.; Odonne, G.; Dayan, L.; Duplais, C.; Espindola, L. S.; Stien, D. Antimicrobial and cytotoxic secondary metabolites from tropical leaf endophytes: Isolation of antibacterial agent pyrrocidine C from Lewia infectoria SNB-GTC2402. *Phytochemistry* **2013**, *96*, 370–377.
477. Lazić, J.; Ajdačić, V.; Vojnovic, S.; Zlatović, M.; Pekmezovic, M.; Mogavero, S.; Opsenica, I.; Nikodinovic-Runic, J. Bis-guanylhydrazones as efficient anti-Candida compounds through DNA interaction. *Appl. Microbiol. Biotechnol.* **2018**, 1–13.
478. Armstrong-James, D.; Brown, G. D.; Netea, M. G.; Zelante, T.; Gresnigt, M. S.; Van De Veerdonk, F. L.; Levitz, S.

M. Series Fungal infections 6 Immunotherapeutic approaches to treatment of fungal diseases. *Lancet Infect. Dis.* **2017**.
479. Kaplancıklı, Z. A.; Levent, S.; Osmaniye, D.; Sağlık, B. N.; Çevik, U. A.; Çavuşoğlu, B. K.; Özkay, Y.; Ilgın, S. Synthesis and Anticandidal Activity Evaluation of New Benzimidazole-Thiazole Derivatives. *Molecules* **2017**, *22*, 2051.
480. Montes, H. M.; Bonifaz, L.; Taborda, C. P.; Nosanchuk, J. D. Editorial: Vaccines, Immunotherapy and New Antifungal Therapy against Fungi: Updates in the New Frontier. *Front. Microbiol* **2017**, *8*.
481. Pfaller, M. A.; Messer, S. A.; Rhomberg, P. R.; Castanheira, M. CD101, a long-acting echinocandin, and comparator antifungal agents tested against a global collection of invasive fungal isolates in the SENTRY 2015 Antifungal Surveillance Program. *Int. J. Antimicrob. Agents* **2017**, *50*, 352–358.
482. Dae Jin, K.; Min Woo, L.; Jeong Su, C.; Seung Gwan, L.; Jee Yoon, P.; Suhng Wook, K. Inhibitory activity of hinokitiol against biofilm formation in fluconazole-resistant Candida species. *PLoS One* **2017**, *12*, e0171244.
483. Heinisch, J. J.; Rodicio, R. Protein kinase C in fungi—more than just cell wall integrity. *FEMS Microbiol. Rev.* **2018**, *42*.
484. Scorzoni, L.; de Paula e Silva, A. C. A.; Marcos, C. M.; Assato, P. A.; de Melo, W. C. M. A.; de Oliveira, H. C.; Costa-Orlandi, C. B.; Mendes-Giannini, M. J. S.; Fusco-Almeida, A. M. Antifungal Therapy: New Advances in the Understanding and Treatment of Mycosis. *Front. Microbiol.* **2017**, *8*, 1–23.
485. Mietton, F.; Ferri, E.; Champleboux, M.; Zala, N.; Maubon, D.; Zhou, Y.; Harbut, M.; Spittler, D.; Garnaud, C.; Courçon, M.; Chauvel, M.; d'Enfert, C.; Kashemirov, B. A.; Hull, M.; Cornet, M.; McKenna, C. E.; Govin, J.; Petosa, C. Selective BET bromodomain inhibition as an antifungal therapeutic strategy. *Nat. Commun.* **2017**, *8*, 15482.
486. Tsai, H.; Bobek, L. Human salivary histatins: promising antifungal therapeutic agents. *Crit. Rev. Oral Biol. Med.* **1998**, *9*, 480–497.
487. Oppenheim, F.; Xu, T.; McMillian, F.; Levitz, S.; Diamond, R.; Offner, G.; Troxler, R. Histatins, a novel family of histidinerich proteins in human parotid secretion. Isolation, characterization, primary structure, and fungistatic effects on Candida albicans. *J. Biol. Chem.* **1988**, *263*, 7472–7477.
488. Edgerton, M.; Koshlukova, S. Salivary histatin 5 and its similarities to the other antimicrobial proteins in human saliva. *Adv. Dent. Res.* **2000**, 14:16–21.
489. Helmerhorst, E.; Hancock, R.; Devine, D.; Oppenheim, F. The antifungal mechanisms of antimicrobial proteins. In *Mammalian antimicrobial proteins*; Hancock, R.; Devine, D., Eds.; Cambridge University PressCambridge University Press, Cambridge, United Kingdom.: Cambridge, United Kingdom, 2004; p. 245–277.

II

Chapter II

Methods to study biofilm matrices of *C. glabrata*

The work presented in this chapter was adapted from:

Rodrigues, C.F.; Silva, S.; Azeredo, J.; Henriques, M. Detection and quantification of fluconazole within *Candida glabrata* biofilms. *Mycopathologia.* **2015**, *179*,(5-6). doi: 10.1007/s11046-015-9862-9.

and presented as poster communications:

Rodrigues, C.F.; Rodrigues, M.E.; Silva, S.; Azeredo, J.; Henriques, M. The chemical structure of the biofilm matrices of *Candida glabrata* induce resistance to antifungal drugs. Biofilms 7 - Microbial Works of Art. **2016**, Porto, Portugal, June 26-28.

Rodrigues, C.F.; Rodrigues, M.E.; Silva, S.; Henriques, M. How do *C. glabrata's* biofilms respond to antifungal drugs? 7th Trends in Medical Mycology. Vol. *Mycoses* 58(Suppl. 4). **2015**, Lisbon, Portugal, 9-12 Oct, 70.

II.1

Detection and quantification of fluconazole within *Candida glabrata* biofilms

ABSTRACT

Candida spp. infections are often associated to biofilms and consequent high resistance to most common drugs (e.g. azoles). These resistance mechanisms are not only associated with the biofilm yeast physiology, but also with the presence of a diffusional barrier imposed by the biofilm matrix, however, the real biochemical role of the biofilm components remains very unclear. So, in order to further clarify this issue, we intend to determine, for the first time, fluconazole in biofilms within both supernatants and matrices. *Candida* spp. biofilms were formed in the presence of fluconazole and it was recovered from both supernatant and matrix cell-free fractions. Then, high pressure liquid chromatography was used to identify and quantify the amount of drug that was present in the two fractions. Moreover, this study also showed that the presence of fluconazole in both fractions indicated that the drug administrated did not completely reached the cells, so this phenomenon can easily be associated with lower biofilm susceptibility, since the drug administered did not completely reached the cells.

Keywords: *Candida*, biofilm matrix, antifungal, method, resistance.

II.1.1 Introduction

The incidence of fungal infections has increased meaningfully in the last decades, especially in immunosuppressed and/or hospitalized patients, causing high levels of morbidity and mortality [1]. The rise in antimicrobial resistance and the restricted number of effective antifungal drugs, which still have many side effects, may be responsible for this event, specially related to infections caused by yeasts from the *Candida* genus [2]. In fact, *Candida* spp. are the third most common cause of nosocomial infections and the most common etiologic agent of fungal-related biofilm infections [3,4]. Between the *Candida* spp., *Candida glabrata* is the second most prevalent pathogenic fungal species in humans, after *Candida albicans* [2]. Even though, *C. glabrata* is not capable to produce hyphae, it has a number of virulent factors, comprising secretion of hydrolytic enzymes, adhesion to host cells or to medical devices and biofilm formation [3]. This last and very important capability can occur on the host mucosa and on the medical indwelling surfaces devices, and it involves the production of an extracellular matrix that encloses yeasts' micro-colonies [4]. Biofilms are biological communities with an extraordinary degree of organization, in which microorganisms form structured, coordinated, and functional communities, embedded in a self-created extracellular matrix. Biofilm production is also associated with a high level of antifungal resistance. The ability of *Candida* spp. to form drug-resistant biofilm is an important factor in their contribution to human disease [5–7]. In the widely held view of microbial biofilms, sessile cells within biofilms are less susceptible to antimicrobial agents than planktonic cells, since the development of drug resistance has been linked with an increase in the maturation process[8,9]. Additionally, many authors have questioned whether biofilm matrices interfere in its resistance. Still, it is difficult to overcome this problem, since there is a lack of methodologies which allow the detection of the amount of xenobiotics able to diffuse into matrix and reach biofilm cells.

High-pressure liquid chromatography (HPLC), is a very sensitive technique used to separate, identify and quantify the components in a mixture and it is very useful in biomedical assays. Reverse Phase-HPLC (RP-HPLC) is a particular model of HPLC which operates on the principle of hydrophobic interactions, allowing the measurement of these interactive forces [10]. As several drugs where already identified and quantified using this procedure [11], this methodology was used to detect fluconazole.

Thus, the present work proposes an adapted methodology for the detection and quantification of an antifungal agent (fluconazole) in the biofilm environment, by high-pressure liquid

chromatography, in order to understand its diffusion within the matrix and to contribute to a better understanding of biofilms' tolerance to antifungal agents' phenomena.

II.1.2. Methods

II.1.2.2 Candida *spp. biofilm formation*

Fluconazole (Flu) was kindly provided by Pfizer, S.A. in its pure compound. Aliquots with a final concentration of 1000 mg/L were prepared in dimethyl-sulfoxide and the final dilutions in Sabouraud dextrose broth medium (SDB) (Merck, Darmstadt, Germany).

To validate the method, two strains of *C. glabrata* (534784 and 562123 from the Hospital Escala Braga, Portugal), were used. The identification of all isolates strains was confirmed using CHROMagar™ *Candida* (CHROMagar™, Paris, France) and by PCR-based sequencing using specific primers (*ITS1* and *ITS4*) against the 5.8s subunit gene reference [12]. The PCRs products were sequenced using the ABI-PRISM Big Dye terminator cycle sequencing kit (Perkin Elmer, Applied Biosystems, Warrington, UK). *Candida glabrata* strains were subcultured on Sabouraud dextrose agar (SDA) (Merck, Darmstadt, Germany) for 24 h at 37°C. Cells were then inoculated in Sabouraud dextrose broth (Merck, Darmstadt, Germany) and incubated for 18 h at 37°C under agitation at 120 rev/min. After incubation, the cells were harvested by centrifugation at 3000 *g* for 10 min at 4°C and washed twice with Phosphate Buffered Saline (PBS 0.1 *M*, pH=7.5). Pellets were then suspended in SDB and the cellular density was adjusted to $1x10^5$ cells/mL using a Neubauer counting chamber. Standardized cell suspensions (500 µL) were placed into selected wells of 24-wells polystyrene microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium). As negative control SDB was used without cells and antifungal agent. As positive control cells suspensions were tested without antifungal agent. At 24 h, 250 µL of SDB medium was removed and an equal volume of fresh SDB plus the plus 250 µL of fluconazole (1000 mg/L, 2x concentrated) were added. The plates were incubated at 37°C for more 24 h, a total of 48 h at 120 rpm/min. After the biofilm formation, the medium was aspirated and non-adherent cells removed by washing the biofilms with sterile ultra-pure water [13].

II.1.2.3 Extraction of the antifungal agent from biofilms

After the biofilm formation in the presence of the antifungal agent, each well of the 24-well plate was treated as one single biofilm microecosystem. A diagram summarizing the protocol steps is presented in Figure II.1.1. Firstly, the supernatant from the biofilms was collected (volume= 350

µL), centrifuged at 12000 g during 10 min, filtered with a 0.22 µm filter to remove possible contaminant cells (supernatant fraction). In parallel, the biofilm remaining in the wells was scrapped with 150 µL of sterile water and the suspension was collected, sonicated (Ultrasonic Processor, Cole Parmer) during 10 seconds at 30%, and centrifuged at 12000 g during 10 min [13]. Finally, the resultant supernatant was filtered with a 0.22 µm filter to remove cells (matrix). Sonication time was optimized for this procedure, making colonies formation units counts as controls. The samples were stored at -20°C until the detection and quantification method was executed.

The high-pressure liquid chromatography (HPLC - Varian 9002/ Pro-Star) method was performed using a C18 column (YMC, Inc.). For that, a slightly modified method from Sadasivudu et al [11] was performed. The mobile phase was acetonitrile:water (50:50) (Fisher Chemicals) with a flow of 1 mL/min, during 6 min for each sample, read at 260 nm. Controls of Flu used for the calibration curve were prepared with the mobile phase in the following range: 1.0, 2.0, 4.0, 8.0, 20.0, 40.0 and 100.0 mg/L. The obtained equation was Area = (0.091 x [Flu]) + 0.007, r^2= 0.996.

Each assay was performed with six samples, in three independent assays.

II.1.3. Results and discussion

In the last years, biofilms are gaining much more importance not only in the research field, but also on the clinical practice. As it is known, they confer significant resistance to antifungal therapy, by limiting the penetration of the xenobiotic through the matrix, thus protecting cells [2,9,14]. However, the knowledge on this ground is still scarce, especially in *Candida* spp. biofilms. The fact that biofilms are very complex structures, due to their environment and matrix, make the studies more difficult to execute. Also, it is acknowledged that antifungal agents action in biofilms is poor, and one of the main responsible for that inactivity the extracellular matrix [14]. The mechanisms involved in this phenomena and the pathway taken by the antifungal compounds or the amount of drug that reaches the cells, crossing the biofilm matrix are still unknown [14–16]. So, it is of major importance to verify if the antifungal agents are still in the biofilm environment (supernatant fraction) or if they are retained in the extracellular matrix (matrix fraction).

Therefore, the aim of this work was to detect and quantify the presence of the Fluconazole on *C. glabrata* biofilms, after its isolation from supernatants and matrices (Figure II.1.1).

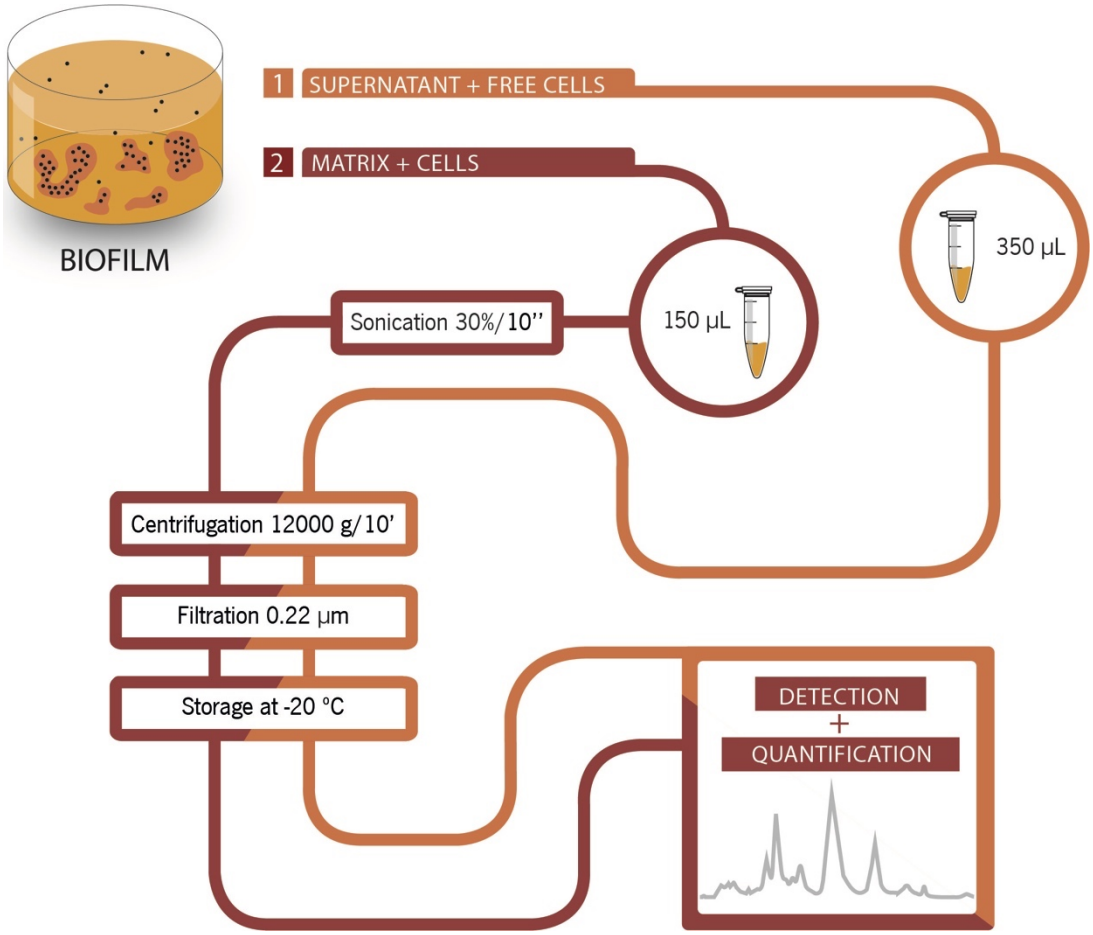


Figure II.1.1. Scheme of the method for detection and quantification of fluconazole within *Candida* spp. biofilms.

The HPLC methodology proposed by Sadasivudu et al [11] was slightly adjusted in order to quantify this drug, recovered from both fractions. As observed in figure II.1.2, Flu was detected in *C. glabrata* biofilm fractions and presented a peak at the retention time of 3.5 minutes, at 260 nm. In addition, the calibration curve obtained (Area = 0.091 [Flu] + 0.007) confirmed that HPLC is a good methodology to detect Flu, with a correlation factor of 0.996. The detection of Flu, present in biofilm supernatants (Figure II.1.2D and II.1.2F) and in their matrices (Figure II.1.2C and II.1.2E), was obtained without any interference, presenting a clear peak with the same retention time.

In order to confirm the reliability of the methodology, two biofilms of *C. glabrata* strains were assessed, since they showed significant different biofilm susceptibilities profiles to Flu ($P<0.0001$): *C. glabrata* 562123 biofilm is more susceptible (20% of biofilm biomass inhibition, using Crystal Violet assay [13]) than *C. glabrata* 534784 biofilm (with 13% of inhibition, from Crystal Violet assay [13]). The quantification of Flu present on both biofilm supernatants and matrices is presented in Table II.1.1.

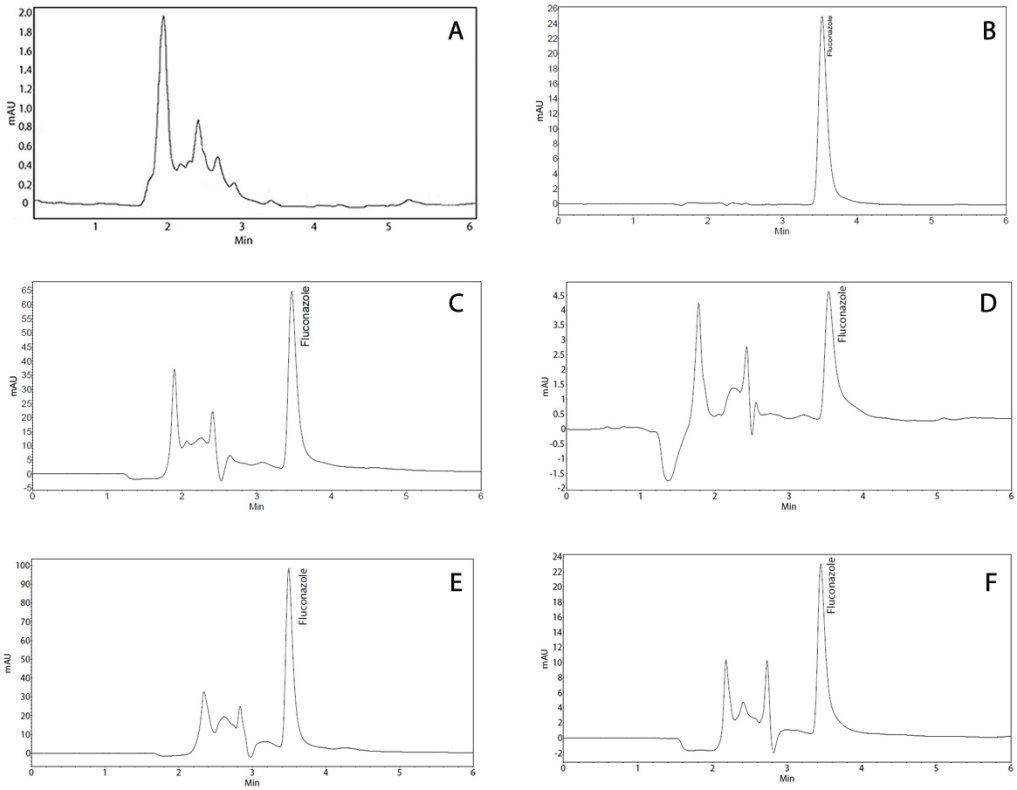


Figure II.1.2 Chromatograms of the detection and quantification of fluconazole within *Candida glabrata* biofilms. (A – Control 0 mg/L; B – Fluconazole's standard 40 mg/L; C – *C. glabrata* 562123 matrix; D – *C. glabrata* 562123 supernantant; E - *C. glabrata* 534784 matrix; F – *C. glabrata* 534784 supernatant.

Table II.1.1 Quantification of Fluconazole (Flu) present in *Candida glabrata* strains biofilm supernatant (S) and matrix (M) fractions

Strains	**Average [Flu] mg/L ± SD**	
C. glabrata 562123	S	551.96 ± 44.22
	M	60.81 ± 1.43
C. glabrata 534784	S	707.29 ± 34.97
	M	67.96 ± 2.54

SD – Standard deviation

Interestingly, in samples of *C. glabrata* 562123, which is the less resistant, we noticed a lower amount of Flu in both fractions, which means that it is likely that there was a higher proportion of the antifungal reaching the cells and, consequently, causing the greater inhibition. In opposition, *C. glabrata* 534784 had a high amount of antifungal in the medium, probably showing the capacity of the strain to avoid the antifungal entering in its cells. It is important to address that the method optimized, has the advantage of detecting low quantities of the drug, is easy and low time consuming. Additionally, this methodology can also be used to study of the dynamic of interaction of other compounds (e.g. hormones, nutrients, and other chemical compounds) within the biofilm matrices of any other microorganism (yeast and bacteria).

In summary, we are proposing a methodology that will allow a better understanding of biofilms and drugs, regarding the amount of a compound present in the supernatant of matrices of biofilm, which will be an important step to understand the problematic of biofilm resistance and the higher number of diseases associated to them.

References

1. Lass-Flörl, C. The changing face of epidemiology of invasive fungal disease in Europe. *Mycoses* **2009**, *52*, 197–205.
2. Silva, S.; Negri, M.; Henriques, M.; Oliveira, R.; Williams, D. W.; Azeredo, J. Candida glabrata, Candida parapsilosis and Candida tropicalis: biology, epidemiology, pathogenicity and antifungal resistance. *FEMS Microbiol. Rev.* **2012**, *36*, 288–305, doi:10.1111/j.1574-6976.2011.00278.x.
3. Pfaller, M. A.; Diekema, D. J. Epidemiology of invasive candidiasis: A persistent public health problem. *Clin. Microbiol. Rev.* **2007**, *20*, 133–163, doi:10.1128/CMR.00029-06.
4. Bassetti, M.; Righi, E.; Costa, A.; Fasce, R.; Molinari, M. P.; Rosso, R.; Pallavicini, F. B.; Viscoli, C. Epidemiological trends in nosocomial candidemia in intensive care. *BMC Infect. Dis.* **2006**, *6*, 21, doi:10.1186/1471-2334-6-21.
5. Hagerty, J. A.; Ortiz, J.; Reich, D.; Manzarbeitia, C. Fungal Infections in Solid Organ Transplant Patients. *Surg. Infect. (Larchmt).* **2003**, *4*, 263–271, doi:10.1089/109629603322419607.
6. Kojic, E. M. E. M.; Darouiche, R. O. R. O. Candida infections of medical devices. *Clin Microbiol Rev.* **2004**, *17*, 255–267., doi:10.1128/cmr.17.2.255-267.2004.
7. Samaranayake, L. P.; Fidel, P. L.; Naglik, J. R.; Al., E. Fungal infections associated with HIV infection. *Oral Dis.* **2002**, *8*, 151–160.
8. Rajendran, R.; Robertson, D. P.; Hodge, P. J.; Lappin, D. F.; Ramage, G. Hydrolytic Enzyme Production is Associated with Candida Albicans Biofilm Formation from Patients with Type 1 Diabetes. *Mycopathologia* **2010**, *170*, 229–235, doi:10.1007/s11046-010-9319-0.
9. DM, K.; MA, G. Candida biofilms: antifungal resistance and emerging therapeutic options. *Curr Opin Investig Drugs.* **2004**, *5*, 186–97.
10. Gerber, F.; Krummen, M.; Potgeter, H.; Roth, A.; Siffrin, C.; Spoendlin, C. Practical aspects of fast reversed-phase high-performance liquid chromatography using 3 µm particle packed columns and monolithic columns in pharmaceutical development and production working under current good manufacturing practice. *J. Chromatogr. A* **2004**, *1036*, 127–133, doi:10.1016/J.CHROMA.2004.02.056.
11. Sadasivudu, P.; Shastri, N.; Sadanandam, M. Development And Validation Of Rp-Hplc And Uv Methods Of Analysis For Fluconazole In Pharmaceutical Solid Dosage Forms. *Int. J. ChemTech Res.* **2009**, *1*, 1131–1136.
12. Williams, D. W.; Wilson, M. J.; Lewis, M. A. O.; Potts, A. J. C. Identification of Candida Species by PCR and Restriction Fragment Length Polymorphism Analysis of Intergenic Spacer Regions of Ribosomal DNA. *J. Clin. Microbiol.* **1995**, *33*, 2476–2479.
13. Silva, S.; Henriques; Martins, A.; Oliveira, R.; Williams, D.; Azeredo, J. Biofilms of non-Candida albicans Candida species: quantification, structure and matrix composition. *Med Mycol.* **2009**, *47*, 681–689.
14. Rodrigues, C. F.; Silva, S.; Henriques, M. Candida glabrata: A review of its features and resistance. *Eur. J. Clin. Microbiol. Infect. Dis.* **2014**, *33*, 673–688, doi:10.1007/s10096-013-2009-3.
15. Prażyńska, M.; Gospodarek, E. In vitro effect of amphotericin B on Candida albicans, Candida glabrata and Candida parapsilosis biofilm formation. *Mycopathologia* **2014**, *177*, 19–27, doi:10.1007/s11046-014-9727-7.
16. Nett, J. E. Future directions for anti-biofilm therapeutics targeting Candida. *Expert Rev. Anti. Infect. Ther.* **2014**, *12*, doi:10.1586/14787210.2014.885838.

II.2
Methods applied to detect the biochemical changes of *Candida glabrata* biofilm matrices

INTRODUCTION

In order to detect and, possibly, identify alterations on polysaccharides, proteins and lipids on the matrices of *C. glabrata* biofilms, in the presence or absence of antifungal drugs (fluconazole, amphotericin B, caspofungin and micafungin), two instrumental methodologies were applied:

- Fourier-transform infrared spectroscopy – attenuated total reflection (FTIR-ATR)
- Matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)

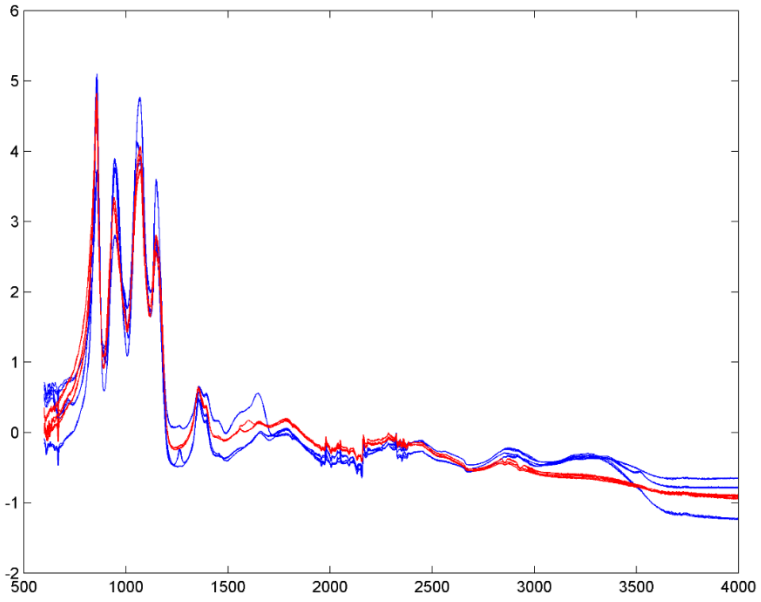
II.2.1. Methods, Results and Discussion

Candida glabrata strains were subcultured on Sabouraud dextrose agar (SDA) (Merck, Darmstadt, Germany) for 24 h at 37°C. Cells were then inoculated in Sabouraud dextrose broth (Merck, Darmstadt, Germany) and incubated for 18 h at 37°C under agitation at 120 rev/min. After incubation, the cells were harvested by centrifugation at 3000 *g* for 10 min at 4°C and washed twice with Phosphate Buffered Saline (PBS 0.1 *M*, pH=7.5). Pellets were then suspended in RPMI-1640 (Sigma-Aldrich, Roswell Park) and the cellular density was adjusted to $1x10^5$ cells/mL using a Neubauer counting chamber. Standardized cell suspensions (1000 µL) were placed into selected wells of 24-wells polystyrene microtiter plates (Orange Scientific, Braine-lAlleud, Belgium). As negative control SDB was used without cells and antifungal agent. As positive control cells suspensions were tested without antifungal agent. At 24 h, 500 µL of RPMI-1640 medium was removed and an equal volume of fresh RPMI-1640 plus 500 µL of antifungals (fluconazole, amphotericin B, caspofungin and micafungin and MBEC concentrations, depending on the strain, 2x concentrated) were added. The plates were incubated at 37°C for more 24 h, a total of 48 h at 120 rpm/min. After the biofilm formation, the medium was aspirated and non-adherent cells removed by washing the biofilms with sterile ultra-pure water[1].

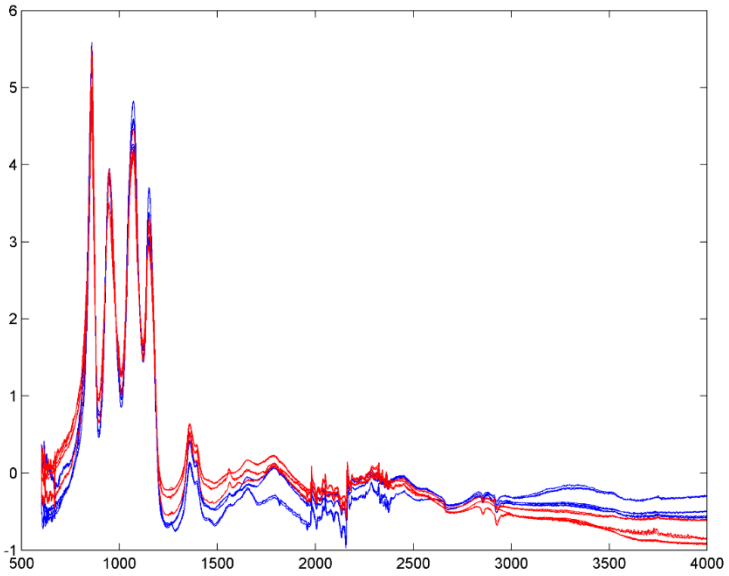
II.2.2. Fourier-transform infrared spectroscopy – attenuated total reflection

FTIR-ATR spectra were acquired at the Faculty of Pharmacy, University of Porto, using a PerkinElmer Spectrum BX FTIR System spectrophotometer, with a PIKE Technologies Gladi ATR accessory from 4000 to 600 cm^{-1} with a resolution of 4 cm^{-1} and 32 scans co-additions.

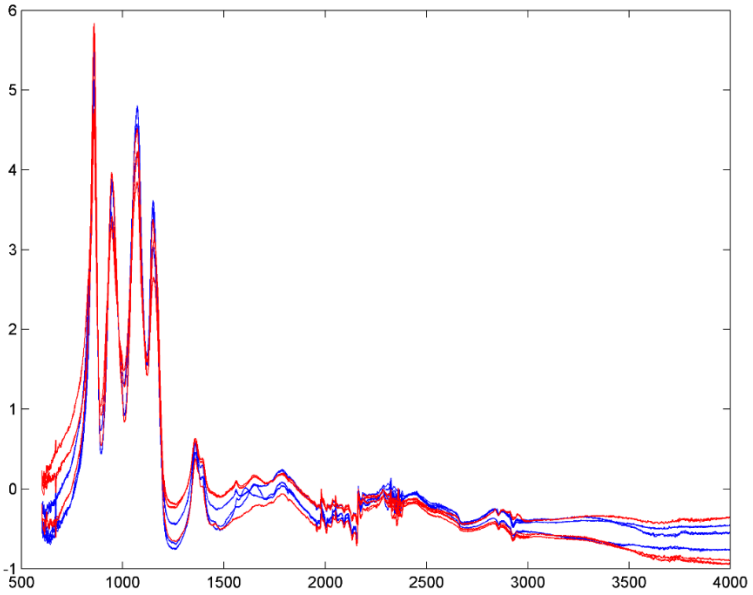
The FTIR-ATR spectra were obtained for the seven clinical isolates studied in this work exhibited the main biomolecules characteristic infrared absorption bands: lipids (3000-2800 cm^{-1}), proteins/amides I and II (1700-1500 cm^{-1}), phospholipids/DNA/RNA (1500-1185 cm^{-1}), polysaccharides (1185-900 cm^{-1}) and the fingerprint region (900-600 cm^{-1}) [2]. It was used 3 to 8 replicates of lyophilized matrix of each clinical isolate (*C. glabrata* AE2, D1, 562123, 534784, 513100, 585626) and reference (*C. glabrata* ATCC2001). This methodology demonstrated to be very sensitive to variations in the samples' composition. Hence, as the biofilms' structure fluctuate too much, even making all efforts to maintain the same conditions of biofilm growing and development (e.g. media, temperature, stress induced, rpm), the matrices composition showed to be too variable to obtain consistency and to characterize, with acceptable error, the percentage of the main biomolecules that composed each isolate (Figure II.2.1).



A



B



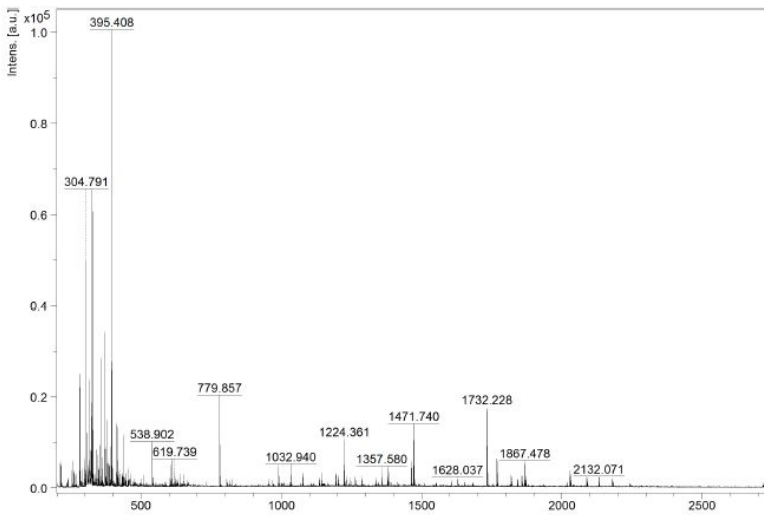
C

Figure II.2.1. FTIR-ATR spectra of in *Candida glabrata* ATCC2001 (A), *Candida glabrata* 562123 (B) and *Candida glabrata* 534784 (C) biofilm matrices of controls in different replicates (colors).

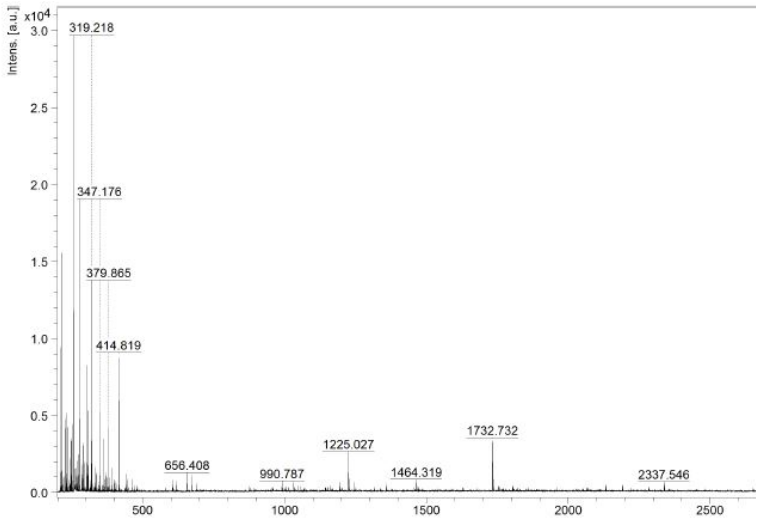
II.2.3. Matrix assisted laser desorption ionization-time of flight mass spectrometry

MALDI-TOF MS was performed on a Bruker Daltonics UltrafleXtreme on the Centre of Materials of the University of Porto (CEMUP). This technique would allow the identification and characterization of compounds on heterogeneous surfaces/mixtures, and the determination of the molecular weight of intact glycoproteins and proteins and amino acid sequencing (proteomics and glycopromatics) [3].

In this case, 3 replicates of the matrix of the reference (*C. glabrata* ATCC2001) were separated and preserved at – 20°C until use. MALDI-TOF MS results also revealed high sensitivity. The variations that the drugs induced in the matrices' compositions were detected and determined, with an acceptable error. Nevertheless, even performing MALDI-TOF-TOF in order to better define the peptides (identification and characterization), the sample demonstrated to be so rich in other biomolecules, that were probable masking the effect of the proteins and peptides in the sample. Using the Mascot Science® software (www.matrixscience.com/) and UniProt (http://www.uniprot.org/), the proteins could not be properly identified. It was concluded that the samples would need to be treated (cleaned from "contaminants"), before analysis, but since the quantity of matrix is always very low, the optimization was provisionally suspended. Even so, differences on the matrices can be easily noticed (Figure II.2.2).



A



B

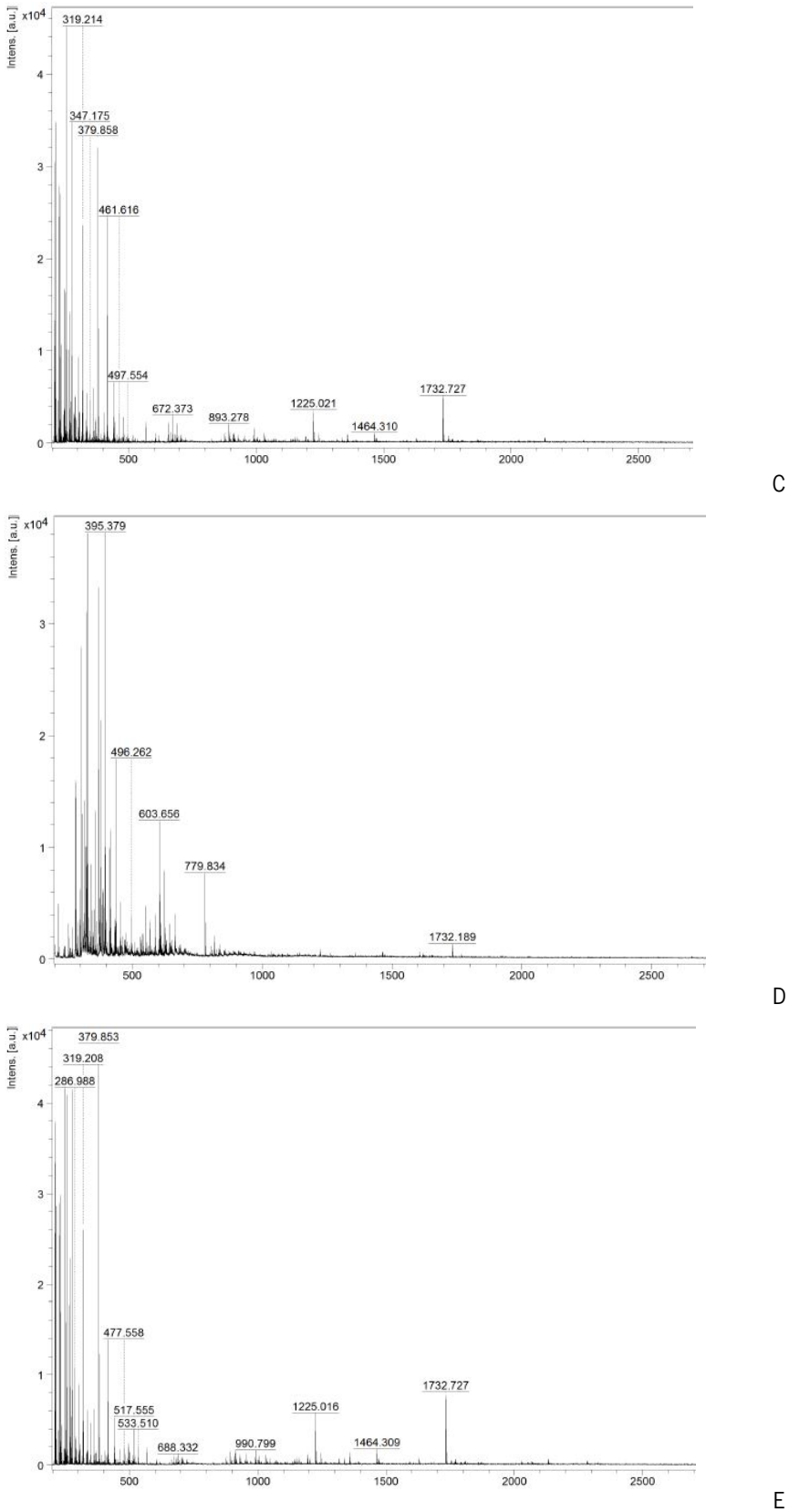


Figure II.2.2. MALDI-TOF-TOF MS spectra of in *Candida glabrata* ATCC2001 biofilm matrices of control (A) and with antifungals (B - Fluconazole; C - Amphotericin B; D - Caspofungin; E - Micafungin).

References

1. Rodrigues, C. F.; Silva, S.; Azeredo, J.; Henriques, M.; Henrique, M.; Henriques, M. Detection and Quantification of Fluconazole Within Candida glabrata Biofilms. *Mycopathologia* **2015**, *179*, 391–5, doi:10.1007/s11046-015-9862-9.
2. Maquelin, K.; Kirschner, C.; Choo-Smith, L.-P.; van den Braak, N.; Endtz, H. P.; Naumann, D.; Puppels, G. J. Identification of medically relevant microorganisms by vibrational spectroscopy. *J. Microbiol. Methods* **2002**, *51*, 255–71.
3. Seneviratne, C. J.; Wang, Y.; Jin, L.; Abiko, Y.; Samaranayake, L. P.; Jayampath Seneviratne, C.; Wang, Y.; Jin, L.; Abiko, Y.; Samaranayake, L. P. Proteomics of drug resistance in candida glabrata biofilms. *Proteomics* **2010**, *10*, 1444–1454, doi:10.1002/pmic.200900611.

III

Chapter III

Candida glabrata biofilms response to azoles and in combination with other compounds

The work presented in this chapter was adapted from:

Rodrigues, C.F.; Gonçalves, B.; Rodrigues, M.E.; Silva, S.; Azeredo, J.; Henriques, M. The Effectiveness of Voriconazole in Therapy of *Candida glabrata*'s Biofilms Oral Infections and Its Influence on the Matrix Composition and Gene Expression. *Mycopathologia*, **2017**, *182*;(7)8. doi: 10.1007/s11046-017-0135-7.

Rodrigues, C.F.; Henriques, M. Oral mucositis caused by *Candida glabrata* biofilms: failure of the concomitant use of fluconazole and ascorbic acid. *Therapeutic Advances in Infectious Diseases*, **2017**, *1*(8). doi: 10.1177/2049936116684477.

and presented as poster communication:

Rodrigues, C.F.; Gonçalves, B.; Rodrigues, M.E.; Silva, S.; Azeredo, J.; Henriques, M. Fluconazole vs Voriconazole: *Candida glabrata*'s biofilms response to different azoles. 7th Trends in Medical Mycology. Vol. *Mycoses* 58(Suppl. 4), **2015**, Lisbon, Portugal, 9-12 Oct, 78-78.

III.1

The effectiveness of voriconazole in therapy of *Candida glabrata* biofilms oral infections and its influence in the matrix composition and gene expression

ABSTRACT

Candida glabrata is one of most prevalent yeast in fungal infections, especially in immunocompromised patients. Moreover, its azole resistance results in a low therapeutic response, particularly when associated to biofilms, as in the esophageal or oropharyngeal candidiasis. The main goal of this work was to study the effectiveness of voriconazole (Vcz) against *C. glabrata* biofilms oral pathologies.

Antifungal susceptibilities were determined in pre-formed 24-hour-biofilms and *ERG* genes expression was determined by qRT-PCR. Protein quantification was performed using BCA Kit®, carbohydrate was estimated according to the Dubois assay and β-1,3-glucans concentration were determined using Glucatell® kit. Finally, ergosterol, voriconazole and fluconazole concentrations within the biofilm matrices were determined by RP-HPLC.

Results showed that *C. glabrata* biofilms were more susceptible to Vcz than to fluconazole (Flu) and *ERG* genes expression evidenced an overexpression of the three *ERG* genes in the presence of both azoles. The matrix content presented a remarked decrease in proteins and an increase in carbohydrates, namely β-1,3-glucans. Ergosterol was successfully detected and quantified in the biofilm matrices, with no differences in all the considered conditions. Vcz demonstrated better diffusion through the biofilms and better cell penetration capacities than Flu, indicating that the structure of the drug molecule fully influences its dissemination through the biofilm matrices.

This work showed that Vcz is notably more effective than Flu for the treatment of resistant *C. glabrata* oral biofilms, which demonstrates a clinical relevance in its future use for the treatment of oropharyngeal/esophaegal candidiasis caused by this species.

Keywords: candidiasis, antifungal, resistance, matrix, voriconazole, *Candida*.

III.1.1. Introduction

The occurrence of fungal infections, as candidiasis and candidemia, has been increasing significantly in the last decades, contributing to high morbidity and mortality. The enhanced use of broad-spectrum antibiotics, catheters and parenteral nutrition, the growth in the number of immunosuppressive diseases, the disruption of mucosal barriers, and the increase in chemotherapy and radiotherapy, are all important risk factors for the development of candidemia [1,2]. *Candida* spp. belong to the normal microbiota of an individual's oral cavity, gastrointestinal, urinary and vaginal tracts [2] and are responsible for numerous clinical manifestations from mucocutaneous overgrowth to bloodstream severe infections [1]. *Candida albicans* is the predominant species in both health and disease conditions, yet, in the last two decades the number of infections due to non-*Candida albicans Candida* (NCAC) spp. increased significantly, particularly those involving *Candida glabrata.* This species lacks some of the virulence factors allied to *Candida* spp. pathogenicity, such as hyphal growth or secret hydrolases [3], but it does not turn out to be less virulent [4,5]. Oropharyngeal/esophaegal candidiasis is a serious oral disease characterized by white patches or plaques (e.g. biofilms of *Candida* spp.) on the tongue and other oral mucous membranes. This infection is usual among immunosuppressed adults, but can also be seen rarely in immunocompetent individuals [6–9].

The ability to form biofilm is one of the most important concerns in *Candida* spp. pathogenicity. Biofilm production can occur on the host mucosa (e.g. mouth, esophagus) and on the surface of medical indwelling devices, involving a matrix that encloses yeasts' micro-colonies in a complex structure [4]. The antifungal biofilm resistance can be inducible in reaction to a drug, or a permanent genetic change resulting from prolonged exposure to that drug. Systematically, this phenomenon comprises changes or over-expression of the spot molecules, active extrusion through efflux pumps, limited diffusion, tolerance, which are all characterized mechanisms used by *Candida* spp. to combat the antifungal treatment effects [4,5].

C. glabrata infections are characterized by an intrinsic low susceptibility to azoles, including the imidazoles (e.g. miconazole) and triazoles (e.g. fluconazole, voriconazole) [10,11]. Voriconazole (Vcz) is a second-generation antifungal agent, which fortunately, presently shows a better *Candida* spp. susceptibility [12–14]. This compound has shown to be a good alternative to treat candidiasis [15–19], in opposition to fluconazole (Flu), which has been largely used for decades, as a prophylactic or therapeutic tretament, inducing drug resistance mechanisms in *Candida* spp. [10,20].

In the 2016's, guidelines for esophageal and oropharyngeal candidiasis and for cases that patients having fluconazole-refractory disease, recommend the use of voriconazole ("strong recommendation; high-quality evidence") [21]. Therefore, this work expects to elucidate if the efficacy of Vcz in the treatment of these biofilm pathologies, specifically involving *Candida glabrata,* the most azole-resistant NCAC. It also aims at an evaluation of the response regarding the genes expression and matrix composition after a contact with this drug.

III.2.1. Material and Methods

III.2.1.1 Organisms

Two clinical isolates of *C. glabrata* recovered from vaginal (*C. glabrata* 534784) and urinary (*C. glabrata* 562123) tracts, from Hospital Escala Braga in Portugal, and one reference strain from the American Type Culture Collection (*C. glabrata* ATCC2001) were used in the course of this study. The identity of all isolates was confirmed using CHROMagar™ *Candida* (CHROMagar™, Paris, France) and by PCR-based sequencing using specific primers (*ITS1* and *ITS4*) against the 5.8s subunit gene reference [22]. Genomic DNA was extracted following previously described procedures [23]. The PCR products were sequenced using the ABI-PRISM Big Dye terminator cycle sequencing kit (Perkin Elmer, Applied Biosystems, Warrington, UK).

III.2.1.2 Growth conditions

For each experiment, strains were subcultured on Sabouraud dextrose agar (SDA) (Merck, Darmstadt, Germany) for 24 h at 37°C. Cells were then inoculated in Sabouraud dextrose broth (SDB) (Merck, Darmstadt, Germany) and incubated for 18 h at 37°C under agitation at 120 rpm. After incubation, the cells were harvested by centrifugation at 3000 *g* for 10 min at 4°C and washed twice with Phosphate Buffered Saline (PBS 0.1 *M*, pH=7.5). Pellets were then suspended in RPMI-1640 (pH=7, Sigma-Aldrich, Roswell Park) or SDA and the cellular density was adjusted to $1x10^5$ cells/mL, using a Neubauer counting chamber.

III.2.1.3 Antifungal drugs

Vcz and Flu were kindly provided by Pfizer®, S.A. in its pure compound. Aliquots of 5000 mg/L were prepared using dimethyl-sulfoxide (DMSO). The final concentrations used were prepared with RPMI-1640 or SDB medium and were: 10, 100 and 1000 mg/L for both drugs.

III.2.1.4 Biofilm susceptibility

Standardized cell suspensions (200 μL) were placed into selected wells of 96-wells polystyrene microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium). RPMI-1640 was used without cells and antifungal agent, as a negative control. As positive control cell suspensions were tested without antifungal agent. At 24 h, 100 μL of RPMI-1640 was removed and an equal volume of fresh RPMI-1640 plus the respective antifungal concentration were added (2x concentrated). The plates were incubated at 37°C for more 24 h, a total of 48 h at 120 rpm.

The number of cultivable cells on biofilms was determined by the enumeration of colony forming units (CFUs) [23]. For that, after the period of biofilm formation, all medium was aspired and the biofilms washed once with 200 μL of PBS to remove non-adherent cells. Then, biofilms were scraped from the wells and the suspensions were vigorously vortexed for 2 min to disaggregate cells from the matrix.

III.2.1.5 Effect of Vcz and Flu on Biofilm matrix

III.2.1.5.1 Extraction method

For matrix analysis biofilms were formed in 24-well polystyrene microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium) [23]. For this, 1000 μL of yeast cell suspension ($1x10^5$ cells/mL in SDB or SDB with Flu and Vcz at 10 and 1000 mg/L) were added to each well and the biofilms performed as described previously. After 24 h, 500 μL of SDB medium was removed and an equal volume of fresh SDB with or without the antifungal agents was added. After 48 h, biofilms were scraped from the 24-well plates, resuspended in ultra-pure water, sonicated (Ultrasonic Processor, Cole-Parmer, Illinois, USA) for 30 s at 30 W, and then the suspension vortexed for 2 min. The suspension was centrifuged at 5000 *g* for 5 min at 4°C and the supernatant filtered through a 0.2 μm nitrocellulose filter. The pellets were dried at 37°C until a constant dry biofilm weight was determined.

III.2.1.5.2 Protein determination

The protein content of the biofilm matrix was measured using the BCA® Kit (Bicinchoninic Acid, Sigma-Aldrich, St Louis, USA), using bovine serum albumin (BSA) as a standard. The values were normalized per g of dry weight of biofilm and presented as mg of protein/g of dry weight of biofilm.

III.2.1.5.3 Carbohydrate determination

Total carbohydrate content of the biofilm matrix was estimated according to the procedure of Dubois et al [24], using glucose as standard. The values were normalized per g of dry weight biofilm and the values presented as mg of carbohydrate/g of dry weight of biofilm.

III.2.1.5.4 β-1,3-glucans concentration determination

The β-1,3-glucans concentrations were determined using Glucatell® kit (Cape Cod®, East Falmouth, USA). The values were normalized per pg/mL of β-1,3-glucans and per total of carbohydrates content.

III.2.1.5.5 Ergosterol determination

For the ergosterol extraction, 2.0 mL of n-hexan (Fisher Chemicals) was added to 10.0 mL of the matrix suspension prepared as previously described and then submitted to vortex during 1 min. This procedure was performed three times and the top solution sequestered to a 10.0 mL amber bottle. After the extraction, the solutions were dried with nitrogen until all the organic solvent evaporated. The dried extract was resupended in 2.0 mL of methanol (Fisher Chemicals), filtered with a 0.45 μm filter and stored at -20°C [25].

The high-pressure liquid chromatography (HPLC - Varian 9002/ Pro-Star) method was performed using a C18 column (YMC, Inc.). An isocratic mobile phase of 100% of methanol (Fisher Chemicals) with a flow of 1 mL/min, during 20 min was used for each sample quantification. The results were automatically revealed by the HPLC detector and then normalized by g of dry weight biofilm determined for each strain and condition [25].

III.2.1.6 Gene expression analysis

III.2.1.6.1Gene selection and primer design for quantitative real-time PCR

Three genes (*ERG3*, *ERG6* and *ERG11*) were selected to study their expression in planktonic and biofilm cells in the absence and presence of both drugs. The gene sequences of interest were obtained from *Candida* Genome Database and the primers for quantitative real-time PCR (qRT-PCR) were designed using Primer 3 (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) web-based software and are listed in Table III.1.1. *ACT1* was chosen to be the housekeeping gene. In order to verify the specificity of each primer pair for its corresponding target gene, PCR products were first amplified from *C. glabrata* ATCC2001.

Table III.1.1 Primers and targets used for the gene expression analysis

Sequence (5'→ 3')	Primer	Target
5'-CAC CCA GTC GGT TAC TT-3'	F	*ERG3*
5'- TTG ACA ACT GGG TTG GA-3'	R	
5'-CTT CGA CAA AGT GTA CGC GA-3'	F	*ERG6*
5'- TAA ACG GCG AAA GTA CCA-3'	R	
5'-CTC CAT ACT TGC CAT TCG-3'	F	*ERG11*
5'-CTT CAG TTG GGT AAC GCC AT-3'	R	
5'- GTT GAC CGA GGC TCC AAT GA-3'	F	*ACT1*
5'- CAC CGT CAC CAG AGT CCA AA-3'	R	

F-forward; R-Reverse

III.2.1.6.2 Preparation of Biofilm cells for RNA extraction

Biofilms of the three strains were grown in 24-wells microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium) in three different conditions – 0 (control), 10 and 1000 mg/L of Flu or Vcz – with a final concentration of $1x10^5$ cells/mL, as described above. After, biofilm formation the medium was aspired and the wells were washed with PBS to remove non-adherent cells. The biofilms were scraped from wells with 1 mL of PBS and sonicated (Ultrasonic Processor, Cole-Parmer, Illinois, USA) for 30 s at 30 W to separate the cells from the biofilm matrix. Cells were harvested by centrifugation at 8000 *g* for 5 min at 4°C.

III.2.1.6.3 RNA extraction

RNA extraction was performed using PureLink RNA Mini Kit (Invitrogen, Carlsbad, USA). Prior to RNA extraction, a lysis buffer (PureLink RNA Mini kit) was prepared adding 1% of *β*-mercaptoethanol. Then, 500 µL of lysis buffer and glass beads (0.5 mm diameter) were added to each pellet. These mixes were homogenized twice for 30 s using a Mini-Bead-Beater-8 (Stratech Scientific, Soham, UK). After cell disruption, the PureLink RNA Mini Kit (Invitrogen, Carlsbad, USA) was used for total RNA extraction according to the manufacturer's recommended protocol. To avoid potential DNA contamination samples were treated with RNase-Free I (Invitrogen, Carlsbad, USA). The RNA extraction was performed at three different independent assays.

III.2.1.6.4 Synthesis of cDNA

To synthesize the complementary DNA (cDNA) the iScript cDNA Synthesis Kit (Bio-Rad, Berkeley, USA) was used according to the manufacturer's instructions. For each sample 10 µL of the

extracted RNA was used at a final reaction volume of 50 µL cDNA synthesis was performed firstly at 70ºC for 5 min and then at 42ºC for 1 h. The reaction was stopped by heating for 5 min at 95ºC.

III.2.1.6.5 Quantitative Real-Time PCR (qRT-PCR)

Real-time PCR (CFX96 Real-Time PCR System; Bio-Rad, Berkeley, USA) was used to determine the relative levels of *ERG3*, *ERG6* and *ERG11* mRNA transcripts in the RNA samples, with *ACT1* used as a reference for *Candida* spp. housekeeping gene. Each reaction mixture consisted of a working concentration of SoFast EvaGreen Supermix (Bio-Rad, Berkeley, USA), 50 µM forward and reverse primers, and 4 µL cDNA, in a final reaction volume of 20 µL. Negative controls (water), as well as, non-transcriptase reverse controls (NRT) were included in each run. The relative quantification of gene expression was performed by the Pfaffl method [26]. Each reaction was performed in triplicate and mean values of relative expression were determined for each gene.

III.2.1.7 Diffusion of Vcz and Flu through the biofilm matrices

III.2.1.7.1 Sample treatment

Biofilm was prepared as explained before, but for a volume of 500 µL. At 24 h, 250 µL were removed and an equal volume of fresh SDB plus the respective antifungal concentration (2 x concentrated) was added. The plates were incubated at 37°C for more 24 h at 120 rpm. After 48 h, first, all medium was collected (approximately 350 µL), centrifuged at 12000 rpm during 10 min, and filtered with a 0.45 µm filter. Then, 150 µL (for a total of 500 µL of cell suspension and SDB used for biofilm production) of sterile water was applied above the biofilm, for scrapping it. The suspension was collected, sonicated during 10 s at 30 W, centrifuged at 12000 rpm during 10 min, and finally filtered with a 0.45 µm filter. The samples were stored at -20ºC until the high-pressure liquid chromatography analysis [27].

III.2.1.7.2 Reverse Phase High-Pressure Liquid Chromatography

The high-pressure liquid chromatography (HPLC – Varian 9002/ Pro-Star) method was performed using a C18 column (YMC, Inc.). An isocratic mobile phase was used for both drugs.

For Vcz, a slightly modified method from Ibrahim et al [28]was used. The mobile phase was 0.05 M disodium hydrogen phosphate buffer (pH 5.5):acetonitrile (1:1, v/v) (Fisher Chemicals) with a flow of 1 mL/min, during 10 min for each sample. The retention time was 7.5 min, at 255 nm.

For Flu, a slightly modified method from Shastri et al [29] was used. The mobile phase was acetonitrile:water (50:50) (Fisher Chemicals) with a flow of 1 mL/min, during 6 min for each sample. The retention time was 3.5 min, at 260 nm.

As the procedure was developed and optimized by the group, all the controls and calibration curves were performed in order to guarantee its applicability.

The results were normalized by g of dry weight biofilm determined for each strain and condition.

III.2.1.8 Statistical Analysis

The experiments were performed in triplicate and in three independent assays. Results were compared using one-way ANOVA, Dunnett's post hoc multiple comparisons test, using GraphPad™ Prism 5™ (CA, USA) software. All tests were performed with a confidence level of 95%.

III.3.1. Results

In order to determine the susceptibility of *C. glabrata* biofilms to Vcz, biofilms were developed during 24h and then incubated other 24 h in the presence of different concentrations of Vcz and Flu (control). Table III.1.2 shows the percentage of cell death (biofilm cell viability) of each *C. glabrata* strain, when biofilms were exposed to 10, 100 and 1000 mg/L of Vcz and Flu concentrations. After a 10 or 100 mg/L treatment with the drugs, neither strains accomplished a reduction of, at least, 50%. On contrary, using 1000 mg/L of Vcz, *C. glabrata* 562123 reached a very good biofilm cell reduction of 70.57%, whereas *C. glabrata* ATCC2001 reached 57.32%, and *C. glabrata* 534784 showing a percentage of 43.37%. As expected, a different behavior occurred with Flu for all strains: this drug was unable to eradicate *C. glabrata* viable biofilm cells, even using the higher concentration (1000 mg/L) (Table III.1.2). In fact, the higher reduction was noticed for *C. glabrata* 534784, merely 5.56%. This condition highlights the pronounced difference between a second-generation azole (Vcz) and a long-standing azole (Flu).

As it is well known, oral biofilms resistance to antifungal agents can be due to both cell genetic alterations and the presence of the biofilm matrix. Therefore, an evaluation of the influence of both agents in the expression of the genes (*ERG*) involved in the biosynthesis of ergosterol (the target of azoles), a well-known mechanism of azoles' resistance was ensuing performed [30,31] and results are shown in Figure III.1.1.

In general, the results demonstrate that the *ERG* gene expression levels are gene, strain, dose and azoles' molecule dependent, with significant differences ($P<0.05$, $P<0.001$ and $P<0.0001$). *C.*

glabrata ATCC2001, showed a visible lower n-fold expression for all *ERG* genes (Figure III.1.1) compared to the two isolate strains.

Table III.1.2. Percentage of cell death found in biofilms of *C. glabrata* strains treated with different concentrations of voriconazole in comparison with cells exposed to fluconazole

***C. glabrata* strain**	**[Drugs] mg/L**	**% Biofilm cell death**	
		Vcz	**Flu**
ATCC2001	10	0.62	0.75
	100	1.71	2.34
	1000	**57.32**	2.22
562123	10	0	0
	100	0	0
	1000	**70.57**	2.18
534784	10	0	3.54
	100	0	4.62
	1000	43.37	5.56

Bold: cell death higher than 50%.

Table III.1.3. β-1,3-glucans/carbohydrates ratio and ergosterol concentration on biofilm matrices of *Candida glabrata* strains in the presence of voriconazole and fluconazole

***C. glabrata* strain**	**[Drug] mg/L**	**[β-1,3 glucans]/Carbohydrates ratio Vcz**	**[β-1,3 glucans]/Carbohydrates ratio Flu**	**[Ergosterol] mg/g biofilm ± SD Vcz**	**[Ergosterol] mg/g biofilm ± SD Flu**
ATCC2001	0	$1.2x10^{-4}$		1.17 ± 0.70	
	10	$1.8x10^{-4}$	$9.8x10^{-4}$	0.58 ± 0.21	1.15 ± 0.77
	1000	$1.1x10^{-4}$	$7.4x10^{-4}$	0.79 ± 0.16	0.51 ± 0.14
562123	0	$1.3x10^{-4}$		1.48 ± 0.71	
	10	$1.7x10^{-4}$	$8.8x10^{-4}$	0.95 ± 0.47	1.01 ± 0.09
	1000	$9.0x10^{-5}$	$4.3x10^{-4}$	1.45 ± 0.30	1.39 ± 0.25
534784	0	$1.2x10^{-4}$		1.32 ± 0.67	
	10	$2.7x10^{-4}$	$11.4x10^{-4}$	1.04 ± 0.21	0.75 ± 0.05
	1000	$1.7x10^{-4}$	$7.5x10^{-4}$	2.16 ± 1.01	1.45 ± 0.07

SD - standard deviation

Regarding the study of the biofilm matrix composition and according to the previous results, *C. glabrata* biofilms were treated with 10 and 1000 mg/L of Vcz and then compared with the control (Flu). In order to obtain additional insights into the causes of the increase of *C. glabrata* oral biofilms resistance to azoles and the differences found between the two agents, the biofilm matrices were analyzed in terms of the most important compounds, namely total carbohydrates, β-1,3-glucans, proteins and ergosterol (Figure III.1.2 and Tables III.1.2 and III.1.3).

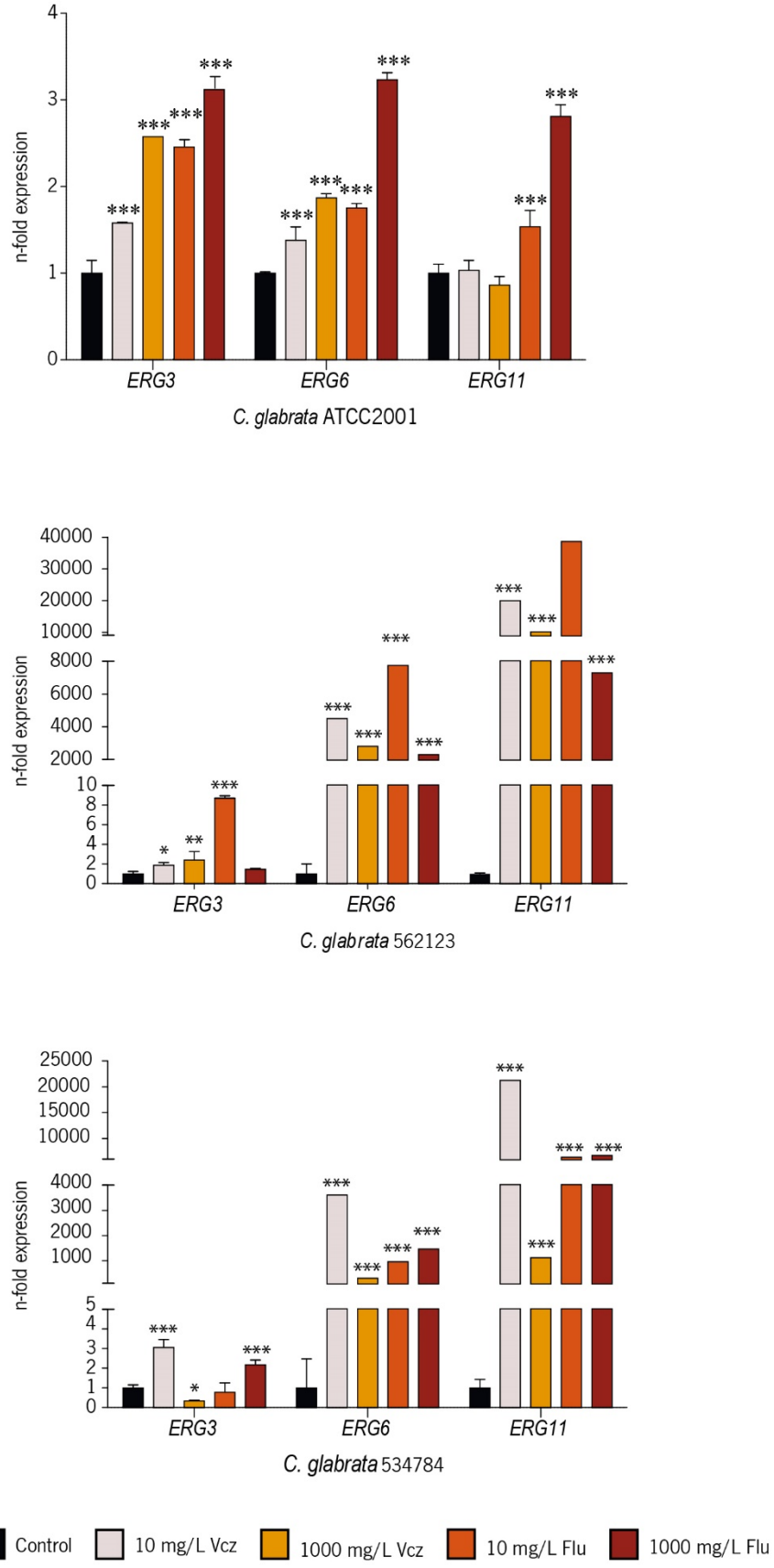


Figure III.1.1. *ERG3*, *ERG6* and *ERG11* n-fold expressions in biofilm cells for each strain, with different concentrations of voriconazole and fluconazole *(* P<0.05; ** P<0.001; *** P<0.0001).*

Notably, a decrease was observed in protein quantities with an increase in the amount of carbohydrates, in the presence of both azoles. This pattern was specially noted for *C. glabrata* 562123 (Figure III.1.2). Overall, the quantity of carbohydrates in the presence of Vcz was a bit lower than in presence of Flu for the three strains.

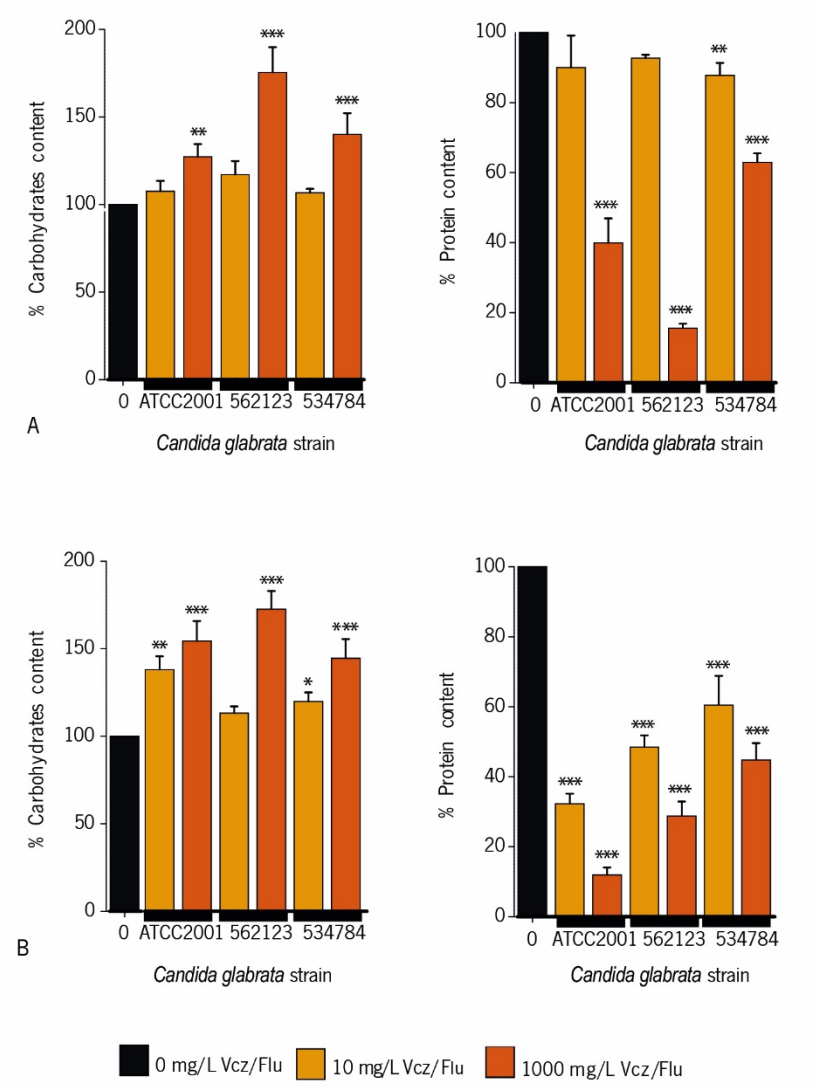


Figure III.1.2. Content of carbohydrates and proteins content in biofilm matrices of *Candida glabrata* strains treated with different concentrations of voriconazole (A) and fluconazole (B) *(* P<0.05; ** P<0.001; *** P<0.0001).*

The quantification of proteins in biofilm matrices (Figure III.1.2), showed a significant decrease in biofilms grown with Vcz and Flu. The higher effect was observed for *C. glabrata* ATCC2001, with Flu application, and the lower effect for *C. glabrata* 534784, for both drugs. It is essential to report that this effect was more perceptible for biofilms grown with Flu, especially for the lower concentration of antifungal agent tested (10 mg/L).

Table III.1.4. Antifungal drug diffusion through *Candida glabrata* biofilm matrices and supernatant

Drug	*C. glabrata* strain	[Drug] matrix (mg/L) ± SD	[Drug] supernatant (mg/L) ± SD
Voriconazole	ATCC2001	0.44 ± 0.20	5.38 ± 1.20
	562123	0.43 ± 0.21	5.27 ± 1.38
	534784	0.60 ± 0.25	5.85 ± 1.67
Fluconazole	ATCC2001	670.80 ± 61.87	69.21 ± 21.49
	562123	551.87 ± 56.31	60.81 ± 20.10
	534784	702.29 ± 77.92	69.88 ± 17.06

SD - standard deviation

Concerning the quantification of total β-1,3-glucans, the results confirm that, in general, there was an increase of total β-1,3-glucans per total carbohydrates, consistent with the previously observed rise of carbohydrates concentration. Afterwards, it was carry out the detection and quantification of ergosterol in biofilms matrices, using HPLC. This lipid was effectively detected but no alterations on its amount noticed in the matrices exposed to both agents (Table III.1.3).

For a final approach, the quantity of the azole agents present in the biofilm matrix and the consequent amount of drug capable of reaching the cells were evaluated. A concentration of 1000 mg/L of each azole was applied in a 24-hour-biofilm for an additional 24 h. Then, the quantity diffused within the biofilm and/or absorbed by the cells was determined according to a protocol described for Flu [27]. Interestingly, the results indicate that Vcz had a higher capacity to penetrate the biofilm net, reaching the cells and eliminating them. As it can be observed in Table III.1.4, Flu was not so effective in all these processes, which may explain its inability to reduce *C. glabrata* oral biofilms, on contrary to the good therapeutic response that Vcz had shown (Table III.1.4).

III.4.1. Discussion

Among the *Candida* spp., *C. glabrata* is the second most prevalent pathogen in humans in the USA and the third in Europe, after *C. albicans* and *C. parapsilosis* [32,33]. *Candida* spp. developed the capability to adapt to different niches and to invade quite a lot of epithelia, namely esophagus, oropharynx or, in even more serious cases, causing septicemia. These infections are very difficult to treat with the traditional antifungal therapies and, consequently, have a high reported morbidity and mortality [34–38].

Agents within the azole class, like Vcz and Flu, are able to vary decisively affecting the spectrum of activity, their pharmacokinetic profiles and toxicities. Vcz and Flu are triazolic derivatives, resulting from replacement of the imidazole ring by triazole, which favors a broad spectrum of action and

selectivity for cytochrome P450 fungal cell. *Candida glabrata* grows only as yeast form *in vivo* and it is believed that the increasing of *C. glabrata* infections is due to its intrinsically low susceptibility to azoles (e.g. Flu) [39–41].

Therefore, the main purposes of this work were to evaluate if Vcz is a good choice to treat, specifically, *C. glabrata* oral biofilms infections, as well to elucidate the mechanisms behind its different responses. Due to its recurrent and traditional use, Flu was used as a control during this study.

Regarding the susceptibility tests of *C. glabrata* biofilms, results show that the two lower concentrations (10 and 100 mg/L) were not effective for the reduction of a minimum of 50% of the biofilm for both drugs. The same happened with 1000 mg/L of Flu. In opposition, with 1000 mg/L of Vcz this reduction was easily reached, noticeably for *C. glabrata* 562123 which had the highest decrease (70.57%). These evidences corroborate the reported facts that (oral) biofilm cells are much more resistant to antifungal agents than planktonic cells [42–47] and are particularly resistant to azoles [48–50].

The relation between the role of the *ERG3*, *ERG6* and *ERG11* expression with the Vcz biofilm's cells susceptibility was evaluated in *C. glabrata* biofilm grown in the presence of Vcz or Flu, by qRT-PCR (Figure III.1.1). *ERG3,* the gene responsible for the conversion of ergosta-5, 7, 24 (28)-trienol to episterol [51] showed an inferior expression for all strains in the presence of Vcz and Flu. *ERG6*, linked to the transformation of fecosterol to zymosterol and *ERG11*, the gene in charge of the production of lanosterol from ignosterol (ergosterol biosynthesis), presented the greater expression in both azoles, remarkably for the isolate strains (*C. glabrata* 562123 and *C. glabrata* 534784). This event occurred probably due to the interference of the azole drugs with the 14α-lanosterol demethilase, obstructing its pathway to produce ergosterol, failing to repose this lipid in the cell membrane [51]. In general, the response was higher for lower concentrations of both drugs (10 mg/L). This assertive reaction to the presence of Vcz and Flu, shows the high ability of *C. glabrata* to rapidly adapt to stress, as it has been showed before, by other authors [52–55], but does not explain the response of *C. glabrata* cells when Vcz is used (and comparing with Flu) in clinical therapeutics. So, the absence of a clear correlation between *ERG* gene expression and the susceptibilities profiles led us to explore the role of the biofilm matrix in azole resistance.

Figure III.1.2 shows a solid decrease in the protein quantification in the presence of both azoles, especially for *C. glabrata* ATCC2001, with the use of Flu (and mostly in the lower concentration used), a phenomenon that may be explained by a response in the stress induced by the drug [56].

On other side, both drugs instigated a minor effect in the loss of proteins, for *C. glabrata* 534784. Contrariwise, biofilms developed with Vcz or Flu, displayed an increase in the amount of carbohydrates (Figure III.1.2), increase that was also confirmed in the quantification of β-1,3-glucans (Table III.1.3). β-1,3-glucans are found in cell wall of *Candida* spp. and also in the biofilm matrix, being linked to the antifungal resistance, by making it difficult to the drugs to diffuse through the biofilm matrices and reach the yeast cells [57–59]. This change in the biofilm matrix composition may be an effort of the cells to obtain a denser matrix, inflexible and more protective for their environment with these biopolymers. Thus, for the obtained results, it is likely that *C. glabrata* biofilm cells are attempting to respond to the azole-caused-stress, producing as many polymers of carbohydrates as possible, aiming to thick the biofilm matrix and, thus, protecting the cells against the drugs.

Additionally, it was questioned if ergosterol, which is a specific *Candida* spp. cell membrane lipid, could be an important factor in the antifungal resistance or *C. glabrata* oral biofilm infections. The results showed that this lipid could not be associated with the resistance or tolerance mechanisms, since, generally, there was no significant variations on its quantity in the biofilm matrices exposed to Vcz or Flu (Table III.1.3).

Finally, the determination of the quantity of the azole agents present in the biofilm matrix and the consequent amount of drug capable of reaching the cells confirmed that Vcz has the capacity to infiltrate better the biofilm matrices than Flu (Table III.1.4). As it is known, Flu has a chemical structure distinct from other antifungal drugs, providing a pharmacokinetic profile highly differentiated with 306.27g/mol. The Vcz chemical structure, although similar to Flu, presents a replacement of one triazole group for a fluoropyrimidine and an introduction of a methyl group. This univalent methyl group shows a stronger hydrophobic interaction with aromatic amino acids and more extensive filling of the substrate-binding site [30], making Vcz more absorbable (96%) than Flu (90%) and with a better connection to the fungi cells, thus emerging as a standard treatment for oropharyngeal or esophageal candidiasis [21]. This was in fact substantiated in this work where *C. glabrata* biofilms are much more susceptible to Vcz than to Flu (Table III.1.4).

Concluding, this study showed that the pattern of *C. glabrata* biofilm susceptibility to Vcz and its comparative molecule, Flu, is effectively different. As it is known, one of the possible mechanisms of resistance to both agents is cell genetic alterations in genes encoding for the ergosterol biosynthesis, which seems to be also the key for the high levels of *C. glabrata* biofilm azoles resistance, but this fact was unable to explain the different resistance profiles obtained for these

two agents. Although there were alterations in the matrix composition (proteins, carbohydrates, including β-1,3-glucans, and ergosterol) in the presence of the azoles, there was not also a direct correlation with Vcz efficacy.

Still, this study demonstrated that the diffusion of the drugs within the biofilms is dependent on the azole used, with Vcz having a better diffusion and an enhanced activity against *C. glabrata* biofilms than Flu, explaining, in part, its better performance and efficacy for oral candidiasis.

Finally, this study also remarks that the tolerance or resistance mechanisms to azoles are multifactorial and cannot be linked to only one feature associated specifically to the cells, the environment or the ecosystems where they persist.

References

1. Eggimann P, Garbino J, P. D. Epidemiology of Candida species infections in critically ill non-immunosuppressed patients. *Lancet Infect Dis.* **2003**, *3*, 685–702.
2. Shao, P.; Huang, L.; Hsueh, P. Recent advances and challenges in the treatment of invasive fungal infections. *Int. J. Antimicrob. Agents* **2007**, *30*, 487–495.
3. Kaur, R.; Domergue, R.; Zupancic, M. L.; Cormack, B. P. A yeast by any other name: Candida glabrata and its interaction with the host. *Curr. Opin. Microbiol.* **2005**, *8*, 378–384, doi:10.1016/j.mib.2005.06.012.
4. Pfaller, M.; Diekema, D. Twelve years of fluconazole in clinical practice: global trends in species distribution and fluconazole susceptibility of bloodstream isolates of Candida. *Clin Microbiol Infect* **2004**, *10*, 11–23.
5. Bethea, E. K.; Carver, B. J.; Montedonico, A. E.; Reynolds, T. B. The inositol regulon controls viability in Candida glabrata. *Microbiology* **2010**, *156*, 452–62, doi:10.1099/mic.0.030072-0.
6. Hasosah MY; Showail, M.; Al-Sahafi, A.; Satti, M.; Jacobson, K. Esophageal candidiasis in an immunocompetent girl. *World J Pediatr* **2009**, *5*, 152–154.
7. Mimidis, K.; Papadopoulos, V.; Margaritis, V.; Thomopoulos, K.; Gatopoulou, A.; Nikolopoulou, V.; Kartalis, G. Predisposing factors and clinical symptoms in HIV-negative patients with Candida oesophagitis: are they always present? *Int J Clin Pr.* **2005**, *59*, 210–213, doi:10.1111/j.1742-1241.2004.00249.x.
8. Marti Cabané, J.; Alvarez Rubio, M. Herpes simplex esophagitis associated to Candida albicans in an immunocompetent host. *An Med Interna* **2004**, *21*, 312.
9. Sathyanarayanan, V.; Razak, A.; Prabhu, M. M.; Saravu, K.; Ganesh, P. C.; Rao, A. K. A case report of herpetic and candidal esophagitis in an immunocompetent adult. *Asian Pac. J. Trop. Biomed.* **2011**, *1*, 251–252, doi:10.1016/S2221-1691(11)60037-3.
10. Xu, S.; Shen, J. Newer antifungal agents micafungin and voriconazole for fungal infection prevention during hematopoietic cell transplantation: a meta-analysis. *1. Xu S, Shen J. Newer Antifung. agents micafungin Vor. fungal Infect. Prev. Dur. Hematop. cell Transplant. a meta-analysis. 2016381-390.* **2016**, *20*, 381–390.
11. Sanguinetti, M.; Posteraro, B.; Fiori, B.; Ranno, S.; Torelli, R.; Fadda, G. Mechanisms of azole resistance in clinical isolates of Candida glabrata collected during a hospital survey of antifungal resistance. *Antimicrob Agents Chemother.* **2005**, *49*, 668–679.
12. Ma, C.; Li, F.; Shi, L.; Hu, Y.; Wang, Y.; Huang, M.; Kong, Q. Surveillance study of species distribution , antifungal susceptibility and mortality of nosocomial candidemia in a tertiary care hospital in China. *BMC Complement. Altern. Med.* **2013**, *13*, 337, doi:10.1186/1471-2334-13-337.
13. Neofytos, D.; Huprikar, S.; Reboli, A.; Schuster, M.; Azie, N.; Franks, B.; Horn, D. Treatment and outcomes of Candida osteomyelitis : review of 53 cases from the PATH Alliance ® registry. *Eur J ClinMicrobiol Infect Dis* **2014**, *33*, 135–141, doi:10.1007/s10096-013-1939-0.
14. Pawar, P.; Kashyap, H.; Malhotra, S.; Sindhu, R. Hp-βCD Voriconazole In Situ Gelling System for Ocular Drug Delivery : In Vitro , Stability , and Antifungal Activities Assessment. *Biomed Res. Int.* **2013**.
15. Chang, T.; Ho, M.; Yang, Y.; Lo, P.; Lin, P.; Wang, A.; Lo, H. Distribution and drug susceptibilities of Candida species causing candidemia from a medical center in central Taiwan. *J Infect Chemother.* **2013**, *19*, 1065–71.
16. Sean, M.; Seenu, M.; William, F.; Gaurav, K.; Michael, D.; Gilbert, M. Management of endogenous fungal endophthalmitis with voriconazole and caspofungin. *Am. J. Ophthalmol.* **2005**, *139*, 135–140.
17. Chen, K.; Wu, W.; Sun, M.; Lai, C.; Chao, A. Endogenous Fungal Endophthalmitis: Causative Organisms,

Management Strategies, and Visual Acuity Outcomes. *Am. J. Ophthalmol.* **2012**, *154*, 213–214.
18. Chang, Y.; Yang, C.; Lee, F.; Lee, S. Voriconazole for Candida endophthalmitis. *Ophthalmology* **2012**, *119*, 2414–2415.
19. Pfaller, M. A.; Messer, S. A.; Rhomberg, P. R.; Jones, R. N.; Castanheira, M. In Vitro Activities of Isavuconazole and Comparator Antifungal Agents Tested agains a Global Collection of Opportunistic Yeasts and Molds. *J. Clin. Microbiol.* **2013**, *51*, 2608–2616, doi:10.1128/JCM.00863-13.
20. Chander, J.; Singla, N.; Sidhu, S.; Gombar, S. Epidemiology of Candida blood stream infections: experience of a tertiary care centre in North India. *J Infect Dev Ctries.* **2013**, *7*, 670–5.
21. Pappas, P. G.; Kauffman, C. A.; Andes, D. R.; Clancy, C. J.; Marr, K. A.; Ostrosky-Zeichner, L.; Reboli, A. C.; Schuster, M. G.; Vazquez, J. A.; Walsh, T. J.; Zaoutis, T. E.; Sobel, J. D. Clinical Practice Guideline for the Management of Candidiasis: 2016 Update by the Infectious Diseases Society of America. *Clin. Infect. Dis.* **2015**, *62*, e1–e50, doi:10.1093/cid/civ933.
22. Williams, D. W.; Wilson, M. J.; Lewis, M. A. O.; Potts, A. J. C. Identification of Candida Species by PCR and Restriction Fragment Length Polymorphism Analysis of Intergenic Spacer Regions of Ribosomal DNA. *J. Clin. Microbiol.* **1995**, *33*, 2476–2479.
23. Silva, S.; Negri, M.; Henriques, M.; Oliveira, R.; Williams, D.; Azeredo, J. Silicone colonization by non- Candida albicans Candida species in the presence of urine. *J. Med. Microbiol.* **2010**, 747–754, doi:10.1099/jmm.0.017517-0.
24. DuBois, M.; Gilles, K.; Hamilton, J.; Rebers, P.; Smith, F. Colorimetric Method for Determination of Sugars and Related Substances. *Anal. Chem.* **1956**, *28*, 350–356, doi:10.1021/ac60111a017.
25. Mar, S.; Morales, H.; Ramos, A. J.; Sanchis, V.; Marin, S.; Morales, H.; Ramos, A. J.; Sanchis, V. Evaluation of growth quantification methods for modelling the growth of Penicillium expansum in an apple-based medium. *J. Sci. Food Agric.* **2006**, *1474*, 1468–1474, doi:10.1002/jsfa.
26. Pfaffl, M. W.; Tichopad, A.; Prgomet, C.; Neuvians, T. P. Determination of stable housekeeping genes , differentially regulated target genes and sample integrity : BestKeeper – Excel-based tool using pair-wise correlations. *Biotechnol. Lett.* **2004**, *26*, 509–515.
27. Rodrigues, C. F.; Silva, S.; Azeredo, J.; Henriques, M.; Henrique, M.; Henriques, M. Detection and Quantification of Fluconazole Within Candida glabrata Biofilms. *Mycopathologia* **2015**, *179*, 391–5, doi:10.1007/s11046-015-9862-9.
28. Ibrahim, A.; Eldin, B.; Ibrahim, A.; Eldin, B.; Shalaby, A. Determination of Voriconazole and its Degradation products in Pharmaceutical formulations using High Performance Liquid Chromatography with Ultra-Violet Detection. *Eurasian J Anal Chem* **2010**, *5*, 254–264.
29. Sadasivudu, P.; Shastri, N.; Sadanandam, M. Development And Validation Of Rp-Hplc And Uv Methods Of Analysis For Fluconazole In Pharmaceutical Solid Dosage Forms. *Int. J. ChemTech Res.* **2009**, *1*, 1131–1136.
30. Fukuoka, T.; Johnson, D.; Winslow, C.; de Groot, M.; C, B.; Hitchcock, C.; Filler, S. Genetic Basis for Differential Activities of Fluconazole and Voriconazole against Candida krusei. *Antimicrob. Agents Chemother.* **2003**, *47*, 1213–1219.
31. Gallagher, J.; Dodds Ashley, E.; Drew, R.; Perfect, J. Antifungal pharmacotherapy for invasive mould infections. *Expert Opin Pharmacother,* **2003**, *4*, 147.
32. de Groot, P. W. J.; Kraneveld, E. a; Yin, Q. Y.; Dekker, H. L.; Gross, U.; Crielaard, W.; de Koster, C. G.; Bader, O.; Klis, F. M.; Weig, M. The cell wall of the human pathogen Candida glabrata: differential incorporation of novel adhesin-like wall proteins. *Eukaryot. Cell* **2008**, *7*, 1951–64, doi:10.1128/EC.00284-08.
33. Fidel, P.; Vazquez, J.; Sobel, J. Candida glabrata: review of epidemiology, pathogenesis, and clinical disease with comparison to C. albicans. *Clin. Microbiol. Rev.* **1999**, *12*, 80–96.
34. Arnold, T.; Dotson, E.; Sarosi, G.; Hage, C. Traditional and emerging antifungal therapies. *Proc Am Thorac Soc.* **2010**, *7*, 222–228.
35. Alcazar-Fuoli, L.; Mellado, E. Current status of antifungal resistance and its impact on clinical practice. *Br. J. Haematol.* **2014**, *166*, doi:10.1111/bjh.12896.
36. West, L.; Lowman, D.; Mora-Montes, H.; Grubb, S.; Murdoch, C.; Thornhill, M.; Gow, N.; Williams, D.; Haynes, K. Differential virulence of Candida glabrata glycosylation mutants. *Biol Chem.* **2013**, *288*, 22006–18.
37. Aghdam, M. R. F.; Sund, S. Invasive Esophageal Candidiasis with Chronic Mediastinal Abscess and Fatal Pneumomediastinum. *Am. J. Case Rep.* **2016**, *17*, 466–471, doi:10.12659/AJCR.898053.
38. AB, W.; D, Miranda-Filho de B, N. R.; RS, R.; M, L. K.; LM, P. The resistance to fluconazole in patients with esophageal candidiasis. *Arq Gastroenterol.* **2009**, *46*, 32–7.
39. *European Committee on Antimicrobial Susceptibility Testing – Antifungal agents, Breakpoint tables for interpretation of MICs, Version 8.0, valid from 2015-11-16*;
40. Kowalsdy, S.; Dixon, D. Fluconazole: a new antifungal agent. *Clin. Pharm.* **1990**, *10*, 179–190.
41. Janssen, P.; Vanden, J.; Bossche, H. Mode of action cytochrome P450 monooxygenase inhibitors. Focus on

azole derivates. *Arch. Pharm. Chem. Sci.* **1987**, *15*, 23–40.
42. Ferrari, S.; Sanguinetti, M.; De Bernardis, F.; Torelli, R.; Posteraro, B.; Vandeputte, P.; Sanglard, D. Loss of mitochondrial functions associated with azole resistance in Candida glabrata results in enhanced virulence in mice. *Antimicrob. Agents Chemother.* **2011**, *55*, 1852–60, doi:10.1128/AAC.01271-10.
43. Al-fattani, M. a; Douglas, L. J. Penetration of Candida Biofilms by Antifungal Agents. *Antimicrob Agents Chemother* **2004**, *48*, 3291–3297, doi:10.1128/AAC.48.9.3291.
44. De Luca, C.; Guglielminetti, M.; Ferrario, A.; Calabrò, M.; Casari, E. Candidemia: species involved, virulence factors and antimycotic susceptibility. *New Microbiol.* **2012**, *35*, 459–468.
45. Grandesso, S.; Sapino, B.; Mazzuccato, S.; Solinas, M.; Bedin, M.; D'Angelo, M.; Gion, M. Study on in vitro susceptibility of Candida spp. isolated from blood culture. *Infect Med.* **2012**, *20*, 25–30.
46. Lewis, R.; Kontoyiannis, D.; Darouiche, R.; Raad, I.; Prince, R. Antifungal activity of amphotericin B, fluconazole, and voriconazole in an in vitro model of Candida catheter-related bloodstream infection. *Antimicrob Agents Chemother.* **2002**, *46*, 3499–505.
47. Donlan, R.; Costerton, J. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.* **2002**, *15*, 167–193.
48. Osawa, K.; Shigemura, K.; Yoshida, H.; Fujisawa, M.; Arakawa, S. Candida urinary tract infection and Candida species susceptibilities to antifungal agents. *J Antibiot (Tokyo).* **2013**, *66*, 651–4.
49. Narita, A.; Muramatsu, H.; Sakaguchi, H.; Doisaki, S.; et al Correlation of CYP2C19 Phenotype With Voriconazole Plasma Concentration in Children. *J Pediatr Hematol Oncol.* **2013**, *35*, (5):e21.
50. Caira, M.; Alkhamis, K.; Obaidat, R. Preparation and crystal characterization of a polymorph, a monohydrate, and an ethyl acetate solvate of the antifungal fluconazole. *J. Pharm. Sci.* **2004**, *93*, 601–61.
51. Henry, K.; Nickels, J.; Edlind, T. Upregulation of ERG genes in Candida species by azoles and other sterol biosynthesis inhibitors. *Antimicrob Agents Chemother.* **2000**, *44*, 2693–700.
52. Berila, N.; Hyroššová, P.; Subík, J. Oxidative stress response and virulence factors in Candida glabrata clinical isolates. *Folia Microbiol. (Praha).* **2011**, *56*, 116–21, doi:10.1007/s12223-011-0016-2.
53. Ng, T. S.; Desa, M. N. M.; Sandai, D.; Chong, P. P.; Than, L. T. L.; Tzu Shan, N.; Desa, M. N. M.; Sandai, D.; Chong, P. P.; Than, L. T. L. Growth, biofilm formation, antifungal susceptibility and oxidative stress resistance of Candida glabrata are affected by different glucose concentrations. *Infect. Genet. Evol.* **2015**, doi:10.1016/j.meegid.2015.09.004.
54. Cu??llar-Cruz, M.; L??pez-Romero, E.; Ruiz-Baca, E.; Zazueta-Sandoval, R. Differential Response of Candida albicans and Candida glabrata to Oxidative and Nitrosative Stresses. *Curr. Microbiol.* **2014**, *69*, 733–739, doi:10.1007/s00284-014-0651-3.
55. Jandric, Z.; Gregori, C.; Klopf, E.; Radolf, M.; Schüller, C. Sorbic acid stress activates the Candida glabrata high osmolarity glycerol MAP kinase pathway. *Front. Microbiol.* **2013**, *4*, 350, doi:10.3389/fmicb.2013.00350.
56. Miyazaki, T.; Kohno, S. ER stress response mechanisms in the pathogenic yeast Candida glabrata and their roles in virulence. *Virulence* **2014**, *5*, 365–70, doi:10.4161/viru.27373.
57. Taff, H. T.; Nett, J. E.; Zarnowski, R.; Ross, K. M.; Sanchez, H.; Cain, M. T.; Hamaker, J.; Mitchell, A. P.; Andes, D. R. A Candida biofilm-induced pathway for matrix glucan delivery: implications for drug resistance. *PLoS Pathog.* **2012**, *8*, e1002848, doi:10.1371/journal.ppat.1002848.
58. Zarnowski, R.; Westler, W. M.; Lacmbouh, G. A.; Marita, J. M.; Bothe, J. R.; Bernhardt, J.; Sahraoui, A. L. H.; Fontainei, J.; Sanchez, H.; Hatfeld, R. D.; Ntambi, J. M.; Nett, J. E.; Mitchell, A. P.; Andes, D. R. Novel entries in a fungal biofilm matrix encyclopedia. *MBio* **2014**, *5*, 1–13, doi:10.1128/mBio.01333-14.
59. Lewis, R. E.; Viale, P.; Kontoyiannis, D. P. The potential impact of antifungal drug resistance mechanisms on the host immune response to Candida. *Virulence* **2012**, *3*, 368–76, doi:10.4161/viru.20746.

III.2

Oral mucositis caused by *Candida glabrata* biofilms: failure of the concomitant use of fluconazole and ascorbic acid

ABSTRACT

Objectives: Candida glabrata is becoming one of the most prevalent pathogenic yeasts in oral diseases. Mucositis is an oral recurrent infection between immunocompromised patients and the actual guidelines recommend the use of fluconazole for many particular cases. However, the azole resistance within *C. glabrata* is renowned, causing a reduced therapeutic response, particularly when associated to biofilms. In this study, we performed an *in vitro* evaluation of an alternative pharmacotherapy for *C. glabrata* biofilm infections, associating ascorbic acid to fluconazole. The ascorbic acid is recognized for degrading β-glucans, one important compound of the biofilm matrices, which are related to the difficulty in drug diffusion.

Materials and Methods: Routine clinical 30 or 40 mg/L doses of fluconazole were applied to *C. glabrata* biofilms simultaneously with 200 or 300 mg/L of ascorbic acid.

Results: The results showed that this association effectively promoted the degradation of the biofilm network but, unfortunately, also stimulated the growth of the yeasts population due to several glucose monomers released by some β-glucans hydrolysis.

Conclusions: As a result, it was proven that, contrarily to what happen in bacteria infections' treatment, ascorbic acid should not be used together with fluconazole in the eradication of *Candida* spp. oral mucositis.

Keywords: *Candida glabrata*, infection, biofilm matrix, fluconazole, ascorbic acid, resistance.

III.2.1. Introduction

Fungal infections are a major clinical health problem that afflict more than 300 million people annually [1]. *Candida glabrata* is a commensal yeast living in human mucosal surfaces (*e.g* mouth, esophagus, intestine) but easily turns into a pathogen, especially in immnunocompromised individuals, instigating a high rate of morbility and mortality [2–5]. In clinical practice *C. glabrata* is the second most prevalent pathogen in humans, in the United States of America, and the third in Europe, after *C. albicans* and *C. parapsilosis* [3,6]. This species is highly resistant to antifungal agents [7–9] and has high capacity to produce biofilms [10–12], which are extremely refractory to antimicrobial therapy, thus being very difficult to treat with the traditional antifungal therapies [10,13–20]. The capability of *Candida* spp. to form biofilms in mouth and/or esophagus mucosae is very common, especially regarding *C. glabrata* [21–23] being a very important feature in the contribution to human oral disease, due to a high level of antifungal resistance [24–26]. Biofilms are biological communities with an extraordinary degree of organization, in which microorganisms form structured, coordinated, and functional communities, embedded in an extracellular matrix [24–26]. These structures are known to induce high rates of morbidity and mortality, especially in immunocompromised patients [27–29], creating the dangerous prospect of ineffective therapies against *Candida* spp. infectious diseases. The National Institutes of Health (NIH) signposts that biofilms are directly or indirectly responsible for over 80% of all microbial infections [30–32]. Therefore, there is an urgent need to overcome the numbers related to biofilm associated *C. glabrata* infections and of major importance to understand the mechanisms behind the response of *C. glabrata* biofilm infection to antifungal treatment.

Mucositis (or oropharyngeal candidiasis) is a frequent infection among immunocompromised patients [33,34], that is characterized by the presence of creamy, white plaques on the tongue and buccal mucosa that generally leave a raw, painful, and ulcerated surface when scraped. Although normally not being a severe infection, it can be uncomfortable and detrimental to the patient's nutritional status thanks to the diminished food and liquid intake. The latest 2016's guidelines recommend the treatment with oral fluconazole (Flu) for general mucositis infections [35]. However, the number of effective antifungal drugs are increasingly limited, and resistance to frequently used agents is emerging [24]. The resistance of *C. glabrata* to the azole's class has been well described [25].

Regarding *Candida* spp. biofilm matrix composition, it is known that it is mainly composed by exopolysaccharides, and the most predominant are β-glucans. These D-glucose-based

polysaccharides are present in different types of glycosidic bonds [26,36–38], consisting of a repeating structure with the β-D-glucose units linked together in linear chains by β-bonds, forming homoglucans, which can extend either from carbon 1 of one saccharide ring to carbon 3 of the next (β-1→ 3) or from carbon 1 to carbon 6 (β-1→ 6). This net of polymers has already shown responsibility by hampering the diffusion of azoles through the biofilms and, thus, from reaching cells inside them[39,40].

The ascorbic acid (AA), also designated as vitamin C, has being highly used concomitantly with antibiotherapy for its capacity to boost the immune system and role as a helper in the treatment of several infections, for several years [41]. AA's concentration in phagocytes and lymphocytes is very high compared with the level in plasma, indicating that AA may have functional roles in these immune system cells, e.g. increasing the functioning of phagocytes, the proliferation of T-lymphocytes and the production of interferon, and decreasing the replication of viruses [42]. Furthermore, its metabolism is affected by various infections (e.g. common cold, pneumonia resultant from many microorganisms, scurvy or *Helicobacter pylori* infections), proposing that there might be treatment benefits in the association of this compound to the pharmacotherapy [43–46]. But also, AA has the capacity of inducing hydroxyl radicals (OH·), which are reactive oxygen species (ROS) that may oxidize biomolecules such as proteins, DNA and other biopolymers (as β-glucans). So, the goal of this study was to check if the *in vitro* efficacy, already demonstrated on the use of AA with antibiotics, will also be the same with an antifungal agent. Therefore, AA will be used concomitantly with Flu to treat *C. glabrata* biofilms oral infections.

III.2.1 Materials and Methods

III.2.1.1 Organisms and growth conditions

Candida glabrata ATCC2001 (reference strain from the American Type Culture Collection) was used in the course of this study. For each experiment, *C. glabrata* ATCC2001 was subcultured on Sabouraud dextrose agar (SDA) (Merck, Darmstadt, Germany) for 24 h at 37°C. Cells were then inoculated in Sabouraud dextrose broth (SDB) (Merck, Darmstadt, Germany) and incubated for 18 h at 37°C under agitation at 120 rpm. After incubation, cells were harvested by centrifugation at 3000 *g* for 10 min at 4°C and washed twice with Phosphate Buffered Saline (PBS 0.1 *M*, pH=7.5). Pellets were then suspended in RPMI-1640 (pH=7, Sigma-Aldrich, Roswell Park) and the cellular density was adjusted to $1x10^5$ cells/mL using a Neubauer counting chamber [47].

III.2.1.2 Fluconazole and ascorbic acid

Fluconazole (Flu) was kindly provided by Pfizer®, S.A.. Ascorbic acid (AA) was purchased from Sigma® (Sigma-Aldrich, Roswell Park). Aliquots of 5000 mg/L were prepared using dimethyl-sulfoxide (DMSO) and the final concentrations used (30 and 40 mg/L for Flu and 200 and 300 mg/L for AA) were prepared with RPMI-1640. Controls were performed with DMSO in order to assure that the concentration used was not toxic (concentrations below 1% (v/v) DMSO).

III.2.1.3 Fluconazole and/or ascorbic acid effect on Candida glabrata *biofilm*

III.2.1.3.1 Biofilm formation

Cell suspension were grown and washed as described above and resuspended in RPMI-1640. Then, standardized cell suspensions (200 μL) were placed into selected wells of 96-wells polystyrene microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium). As a negative control RPMI-1640 was used without cells and antifungal agent. As positive control cell suspensions were tested without antifungal agent. After 24h, 100 μL of RPMI-1640 was removed and an equal volume of fresh RPMI-1640 plus the antifungal agent (2x concentrated) or the association between flu and AA was used. The plates were incubated at 37°C for an additional 24 h period, a total of 48 h at 120 rpm. In addition, in order to assess the effect of glucose in the medium, the assay was performed in the same conditions explained above using RPMI-1640 supplemented with 2% of glucose.

III.2.1.3.2 Biofilm cultivable cells and biomass determination.

The number of biofilms' cultivable cells was determined by the enumeration of colony forming units (CFUs). For that, after the period of biofilm formation, all medium was aspired and the biofilms washed once with 200 μL of PBS to remove non-adherent cells. After, biofilms were scraped from the wells and the suspensions were vigorously vortexed for 2 min to disaggregate cells from the matrix. Serial decimal dilutions in PBS were plated on SDA and incubated for 24 h at 37°C. The results were presented as total of CFUs per unit area (Log_{10} CFUs/cm^2). Total biofilm biomass was quantified by crystal violet (CV) staining. After biofilms formation, the medium was aspirated and non-adherent cells removed by washing the biofilms with sterile ultra-pure water. Then, biofilms were fixed with 200 μL methanol, which was removed after 15 min of contact. The microtiter plates were allowed to dry at room temperature, and 200 μL of CV (1% v/v) added to each well and

incubated for 5 min. The wells were then gently washed twice with sterile, ultra-pure water and 200 μL of acetic acid (33% v/v) were added to release and dissolve the stain. The absorbance of the obtained solution was read in triplicate in a microtiter plate reader (Bio-Tek Synergy HT, Izasa, Lisbon, Portugal) at 570 nm. The results were presented as percentage of biomass [47].

III.2.1.4 Statistical Analysis

All the assays were performed in triplicate and on three separate occasions. Results were compared using one-way ANOVA, Dunnett's and Bonferroni's post hoc multiple comparisons tests, using GraphPad™ Prism 5™ (CA, USA) software. All tests were performed with a confidence level of 95%.

III.3.2. Results

The present study aimed to evaluate an alternative treatment of oral mucositis related to *C. glabrata* biofilms using an association of Flu and AA, similarly to what has been used for treatment of bacterial infections for years.

To determine the influence of AA in the fungistatic activity of Flu, *C. glabrata* ATCC2001 biofilms were grown for 24 h and then different concentrations of these agents were added and led to grow for an additional period of 24 h. The work concentrations, for both Flu and AA, were chosen for being used in clinical practice, since the goal was to overtake the biofilm resistance of *C. glabrata*, using Flu in the same doses, but with another compound as an adjuvant.

Figure III.2.1 shows the percentage of biomass of *C. glabrata* ATCC2001 when exposed to different AA combined with Flu, in a pre-formed 24-h-biofilm. Controls were performed with the two agents alone.

After 24 h of exposure, AA alone had no effect on the reduction of the biofilm biomass, compared to the lower dose of Flu. Furthermore, AA showed to be an enhancer of *C. glabrata* biomass (Figure III.2.1). In other hand, combining 30 mg/L of Flu with both concentrations of AA the biomass presented a statistically significant reduction ($P<0.001$), comparing with the use of Flu alone, however in a very low extension: 10% to 20%. Moreover, when the Flu concentration was higher, this effect was not noticed. It was even observed an increase in biofilm biomass in the presence of the higher concentration of AA when used with the 40 mg/L of Flu. In order to explain the results obtained regarding the lack in the drug activity of Flu, cells' viability after treatment was also assessed (Table III.2.1) for the drugs alone.

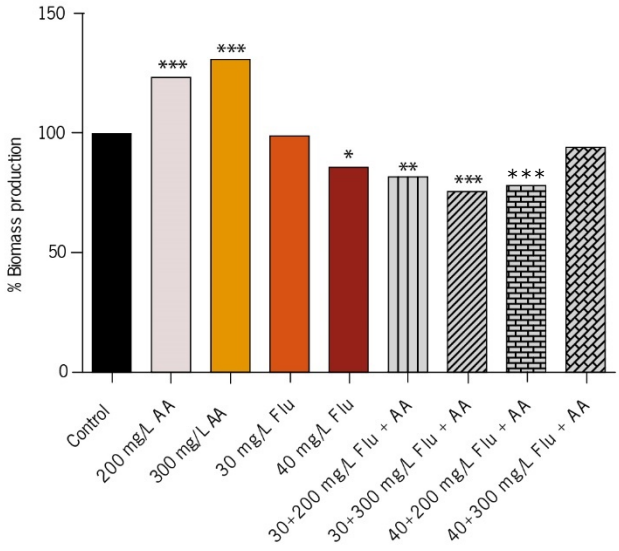


Figure III.2.1. Percentage of biomass detected using crystal violet (CV) staining with Flu and Flu + AA in biofilms of *Candida glabrata* ATCC2001 (* P<0.05; ** P<0.01; *** P<0.001). The control was considered to have 100% of biomass production.

It was verified that using AA, the viable cells increased (around 10% and 15% for 200 and 300 mg/L of AA, respectively), when compared to the controls. Flu was unable to eradicate 50% of *C. glabrata* viable cells even at higher concentrations (Table III.2.1). In fact, when the highest concentration of Flu (40 mg/L) was used, it was only possible to reach a reduction of biofilm cell viability of 7.0% (Table III.2.1). With 30 mg/L the values were rather superior (9.1%), but with no expression in biomass reduction (Figure III.2.1), showing not to be therapeutically interesting.

Table III.2.1. Colony forming units (CFU) count (Log_{10} CFUs/cm^2) when using AA and Flu alone in biofilms of *Candida glabrata* ATCC2001 after 24 h and percentage of CFU reduction

Condition	Log_{10} CFUs/cm^2 ± SD	% CFU reduction
Control	6.22 ± 0.02	–
AA 200 mg/L	6.83 ± 0.25	- 9.8
AA 300 mg/L	7.22 ± 0.05	- 16.08
Flu 30 mg/L	5.65 ± 0.11	9.1
Flu 40 mg/L	5.78 ± 0.48	7.07

SD-standard deviation

To verify if the glucose resultant from the β-glucans hydrolysis is contributing to the growing of the *C. glabrata* population another assay was performed. The RPMI-1640 was supplemented with 2% of glucose for three selected conditions: 200 mg/L of AA, 30 mg/L of Flu and 30 mg/L of Flu + 200 mg/L of AA (Figure III.2.2).

With extra glucose in the medium, the control grew near 10% more than the control without glucose. Also, significant results were obtained when comparing the use of 200 mg/L of AA to 30 and 40 mg/L of Flu alone (P<0.01 and P<0.001, respectively). No significant differences were obtained when comparisons with the drug associations were made (Figure III.2.2).

Lastly, concerning the drug association (30 mg/L Flu + 200 mg/L AA), results show that the presence of glucose (Figure III.2.2) in the medium was clearly harmful, since there was no variation in CFU count (thus, no cell death) comparing with the respective control. Additionally, comparing this result with the control without glucose, there was actually a development in cell population (Figure III.2.2).

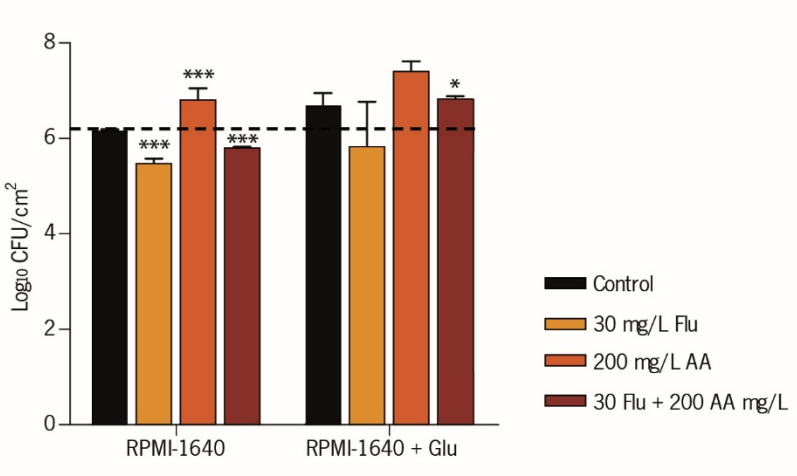


Figure III.2.2. Cell production with Flu and Flu + AA in biofilms of *Candida glabrata* ATCC2001, represented by colony forming units (CFU) count (Log_{10} CFUs/cm^2) (* P<0.05; *** P<0.001).

III.4.2. Discussion

Mucositis associated to erythematous ulcerations in the oral cavity originates pain, xerostomia, dysphagia, and lastly septicaemia [48]. It disturbs functions as drinking, eating, speaking, dental and other mouth care, and it affects not only nutrition and quality but also threatening life [23,48]. Despite the recognized resistant profile to antifungal agents of *Candida* biofilm cells [15,17,18], mucositis still have good outcomes with the use of azoles, particularly fluconazole [23,33,35,49]. The knowledge that the therapeutic response of *C. glabrata* oral biofilms to Flu is lower than the other *Candida* spp. (e.g. *C. parapsilosis* or *C. guillermondii*) [24–26] is the main reason for the development of the present study, in which clinical doses of Flu are associated with AA.

It was verified (Figure III.2.1) that AA isolated had no effect on the reduction of the biofilm biomass, compared to 30 mg/L of Flu and it was shown an increase in the growth of *C. glabrata* biomass.

Generally, no significant differences were obtained when comparisons with the drug associations were made. With the aim of explaining these results, cells' viability after treatment with the drugs alone, was also assessed (Table III.2.1). It was then possible to observe, contrarily to what was expected, an increase in the cell viability, showing a possible degradation and consumption of β-glucans. In fact, other researchers described that during the degradation of AA, the hydrolysis into glucose monomers occurred [50,51]. Xu et al [52], have also showed a similar situation. The authors tested a considered antifungal drug compound, β-1,3-glucanase, in a *Candida albicans* strain, but instead of having reduced in yeast count, had a proliferation in *C. albicans*, which can be explained by the same reasons exposed above. Similarly to the goal of this work, Al-Fattani and Douglas [53] tested various enzymes to degrade biofilms and noted that they were also easily detached from biofilms, but, as specific hydrolase-enzymes, they released many glucose monomers. Despite this fact and since the authors used amphotericin B as antifungal agents, the results in the CFU count were, yet, favorable.

The chemical events underlying this process were explained through clarifications of the β-glucan degradation pathways [54–57]. Thereby, there are, mainly, two paths: (i) the oxidative cleavage of β-glucan is initiated by the removal of a hydrogen atom from the anomeric carbon (C1) of the polysaccharide inducing the formation of an alkyl radical and there are two possible courses for the cleavage of the glycosidic bond with the generation of a lactone in C1. The glycosidic bond may fragment, due to delocalization of the unpaired electron, leading to the release of a β-glucan fragment with a lactone, and of a β-glucan fragment with an alkyl radical, which may react with O_2 to form the corresponding peroxyl radical, which can further undergo transformations. Also, the glucan with an alkyl radical on the C1 can also suffer hydrolysis, which would point to the release of a non-radical β-glucan fragment (glucose monomers) and a β-glucan fragment containing a radical at C1; (ii) the formation of peroxyl radical following the alkyl radical in C1. The carbon centered radical in C1 would react rapidly with O_2 to give a peroxyl radical, which can further combine with another peroxyl radical and fragment via an alkoxyl radical (R–O·) [45]. The alkoxyl radical formed would undergo a β-fragmentation, liberating a β-glucan fragment with a lactone in C1 and a β-glucan fragment with an alkoxyl radical at C3. Other pathways can contribute to the scission of β-glucan, since the non-selective attack of ·OH radicals most likely also generate radicals at other locations besides C1 [43,58].

In fact, a loss of viscosity in the biofilm was observed during the biofilm manipulation whenever AA was used. The biofilm was more flexible and easily breakable and it has been described as being

related with the formation of hydroxyl radicals in the β-glucan degradation [43,56,59,60]. Actually, the glucose hydrolysis reaction is used as alternative method to the Dubois et al [61] for carbohydrate quantification [62,63].

Regarding the concomitant use of Flu and AA (30 mg/L Flu + 200 mg/L AA), the addition of glucose to the RPMI-1640 was very unfavorable. No cell death was achieved (there was no variation in the CFU count), comparing with the respective control. In fact, there was even a small increase in cell population (Figure III.2.2), related to the consumption of the free glucose derived from the hydrolysis of the β-glucans and/or the supplementation of the medium, which disrupted the Flu fungistatic activity.

To conclude, fluconazole is a drug which conserves significance in the treatment of mucositis. Although with certain side effects, it is generally well tolerated, it has low toxicity and the usual therapeutic regimen is very appealing to the patient. Unfortunately, once more, the final data presented indicate that this drug might not work for infections derived from *C. glabrata* biofilms, as previous work from our group have demonstrated. The matrix of *Candida* spp. biofilms has already confirmed to be a solid barrier to the drugs diffusion to the cells. Thus, the use of a drug association in which one drug would degrade this matrix and the other drug would be fungicidal or fungistatic, could be a good strategy and improve the therapeutic response in fungal infections. Flu has showed to be recalcitrant *in vitro* due to the lack of drug penetration [64].

AA leads to a reaction of β-glucan hydrolysis which originates glucose that are later used as carbon source by *C. glabrata,* enabling the cells to colonize and infect. Thus, their capacity of virulence was unable to be annulled by Flu in this drug association. Though it can be predictable that this outcome might be similar to other *Candida* strains and/or species, this work has a clear limitation by the use of a single strain and cannot be directly generalized to all *C. glabrata* strains.

References

1. Brown, G. D.; Denning, D. W.; Gow, N. A. R.; Levitz, S. M.; Netea, M. G.; White, T. C. Hidden killers: human fungal infections. *Sci. Transl. Med.* **2012**, *4*, 165rv13, doi:10.1126/scitranslmed.3004404.
2. Almirante, B.; Rodríguez, D.; Park, B. J.; Cuenca-Estrella, M.; Planes, A. M.; Almela, M.; Mensa, J.; Sanchez, F.; Ayats, J.; Gimenez, M.; Saballs, P.; Fridkin, S. K.; Morgan, J.; Rodriguez-Tudela, J. L.; Warnock, D. W.; Pahissa, A.; Rodrı, D. Epidemiology and Predictors of Mortality in Cases of Candida Bloodstream Infection : Results from Population-Based Surveillance , Barcelona , Spain , from 2002 to 2003 Epidemiology and Predictors of Mortality in Cases of Candida Bloodstream Infection : Re. *J. Clin. Microbiol.* **2005**, *43*, 1829–1835, doi:10.1128/JCM.43.4.1829.
3. Fidel, P.; Vazquez, J.; Sobel, J. Candida glabrata: review of epidemiology, pathogenesis, and clinical disease with comparison to C. albicans. *Clin. Microbiol. Rev.* **1999**, *12*, 80–96.
4. Kao, A.; Brandt, M.; Pruitt, W.; Conn, L.; Perkins, B.; Stephens, D.; Baughman, W.; Reingold, A.; Rothrock, G.; Pfaller, M.; Pinner, R.; Hajjeh, R. The epidemiology of candidemia in two United States cities: results of a population-based active surveillance. *Clin. Infect. Dis.* **1999**, *29*, 1164–1170, doi:10.1086/313450.
5. Kusne, S.; Tobin, D.; Pasculle, A. W.; Van Thiel, D. H.; Ho, M.; Starzl, T. E. Candida carriage in the alimentary tract of liver transplant candidates. *Transplantation* **1994**, *57*, 398–402.

6. de Groot, P. W. J.; Kraneveld, E. a; Yin, Q. Y.; Dekker, H. L.; Gross, U.; Crielaard, W.; de Koster, C. G.; Bader, O.; Klis, F. M.; Weig, M. The cell wall of the human pathogen Candida glabrata: differential incorporation of novel adhesin-like wall proteins. *Eukaryot. Cell* **2008**, *7*, 1951–64, doi:10.1128/EC.00284-08.
7. Lass-Flörl, C. The changing face of epidemiology of invasive fungal disease in Europe. *Mycoses* **2009**, *52*, 197–205.
8. Krogh-Madsen, M.; Arendrup, M. C. M. C.; Heslet, L.; Knudsen, J. D. J. D. Amphotericin B and caspofungin resistance in Candida glabrata isolates recovered from a critically ill patient. *Clin. Infect. Dis.* **2006**, *42*, 938–944., doi:10.1086/500939.
9. Ellis, D. Amphotericin B: spectrum and resistance. *J. Antimicrob. Chemother.* **2002**, *49 Suppl 1*, 7–10.
10. Rodrigues, C. F.; Silva, S.; Henriques, M. Candida glabrata: A review of its features and resistance. *Eur. J. Clin. Microbiol. Infect. Dis.* **2014**, *33*, 673–688, doi:10.1007/s10096-013-2009-3.
11. Sánchez-Vargas, L. O.; Estrada-Barraza, D.; Pozos-Guillen, A. J.; Rivas-Caceres, R. Biofilm formation by oral clinical isolates of Candida species. *Arch. Oral Biol.* **2013**, *58*, 1318–26, doi:10.1016/j.archoralbio.2013.06.006.
12. Martins, C. H. G.; Pires, R. H.; Cunha, A. O.; Pereira, C. A. M.; Singulani, J. de L.; Abrão, F.; Moraes, T. de; Mendes-Giannini, M. J. S. Candida/Candida biofilms. First description of dual-species Candida albicans/C. rugosa biofilm. *Fungal Biol.* **2016**, *120*, 530–537, doi:10.1016/j.funbio.2016.01.013.
13. Sardi, J. C. O.; Scorzoni, L.; Bernardi, T.; Fusco-Almeida, A. M.; Mendes Giannini, M. J. S. Candida species: Current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. *J. Med. Microbiol.* **2013**, *62*, 10–24, doi:10.1099/jmm.0.045054-0.
14. d'Enfert, C.; Janbon, G. Biofilm formation in Candida glabrata: What have we learnt from functional genomics approaches? *FEMS Yeast Res.* **2016**, *16*, fov111, doi:10.1093/femsyr/fov111.
15. Ferrari, S.; Sanguinetti, M.; De Bernardis, F.; Torelli, R.; Posteraro, B.; Vandeputte, P.; Sanglard, D. Loss of mitochondrial functions associated with azole resistance in Candida glabrata results in enhanced virulence in mice. *Antimicrob. Agents Chemother.* **2011**, *55*, 1852–60, doi:10.1128/AAC.01271-10.
16. Al-fattani, M. a; Douglas, L. J. Penetration of Candida Biofilms by Antifungal Agents. *Antimicrob Agents Chemother* **2004**, *48*, 3291–3297, doi:10.1128/AAC.48.9.3291.
17. De Luca, C.; Guglielminetti, M.; Ferrario, A.; Calabrò, M.; Casari, E. Candidemia: species involved, virulence factors and antimycotic susceptibility. *New Microbiol.* **2012**, *35*, 459–468.
18. Grandesso, S.; Sapino, B.; Mazzuccato, S.; Solinas, M.; Bedin, M.; D'Angelo, M.; Gion, M. Study on in vitro susceptibility of Candida spp. isolated from blood culture. *Infect Med.* **2012**, *20*, 25–30.
19. Lewis, R.; Kontoyiannis, D.; Darouiche, R.; Raad, I.; Prince, R. Antifungal activity of amphotericin B, fluconazole, and voriconazole in an in vitro model of Candida catheter-related bloodstream infection. *Antimicrob Agents Chemother.* **2002**, *46*, 3499–505.
20. Donlan, R.; Costerton, J. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.* **2002**, *15*, 167–193.
21. Kamikawa, Y.; Mori, Y.; Nagayama, T.; Fujisaki, J.; Hirabayashi, D.; Sakamoto, R.; Hamada, T.; Sugihara, K. Frequency of clinically isolated strains of oral Candida species at Kagoshima University Hospital, Japan, and their susceptibility to antifungal drugs in 2006-2007 and 2012-2013. *BMC Oral Health* **2014**, *14*, 14, doi:10.1186/1472-6831-14-14.
22. Tati, S.; Davidow, P.; McCall, A.; Hwang-Wong, E.; Rojas, I. G.; Cormack, B.; Edgerton, M. Candida glabrata Binding to Candida albicans Hyphae Enables Its Development in Oropharyngeal Candidiasis. *PLOS Pathog.* **2016**, *12*, e1005522, doi:10.1371/journal.ppat.1005522.
23. Westbrook, S. D.; Kirkpatrick, W. R.; Wiederhold, N. P.; Freytes, C. O.; Toro, J. J.; Patterson, T. F.; Redding, S. W. Microbiology and epidemiology of oral yeast colonization in hemopoietic progenitor cell transplant recipients. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol.* **2013**, *115*, 354–358, doi:10.1016/j.oooo.2012.10.012.
24. Samaranayake, L. P.; Fidel, P. L.; Naglik, J. R.; Al., E. Fungal infections associated with HIV infection. *Oral Dis.* **2002**, *8*, 151–160.
25. Kojic, E. M.; Darouiche, R. O. Candida infections of medical devices. *Clin Microbiol Rev.* **2004**, *17*, 255–267.
26. Usui, S.; Tomono, Y.; Sakai, M.; Kiho, T.; Ukai, S. Preparation and antitumor activities of beta-(1→6) branched (1→3)-beta-D-glucan derivatives. *Biol Pharm Bull.* **1998**, *18*, 1630–1636.
27. Arnold, T.; Dotson, E.; Sarosi, G.; Hage, C. Traditional and emerging antifungal therapies. *Proc Am Thorac Soc.* **2010**, *7*, 222–228.
28. Alcazar-Fuoli, L.; Mellado, E. Current status of antifungal resistance and its impact on clinical practice. *Br. J. Haematol.* **2014**, *166*, doi:10.1111/bjh.12896.
29. West, L.; Lowman, D.; Mora-Montes, H.; Grubb, S.; Murdoch, C.; Thornhill, M.; Gow, N.; Williams, D.; Haynes, K. Differential virulence of Candida glabrata glycosylation mutants. *Biol Chem.* **2013**, *288*, 22006–18.
30. CJ, N.; AD, J. Candida albicans biofilms and human disease. *Annu Rev Microbiol* **2015**, *69*, 71e92.
31. Fox EP, N. C. A sticky situation: untangling the transcriptional network controlling biofilm development in Candida

albicans. *Transcription* **2012**, *3*, 315–22.
32. Gulati, M.; Nobile, C. J. Candida albicans biofilms: Development, regulation, and molecular mechanisms. *Microbes Infect.* **2015**, *18*, 310–321, doi:10.1016/j.micinf.2016.01.002.
33. Lefebvrea, J.-L.; Domengeb, C. A comparative study of the efficacy and safety of fluconazole oral suspension and amphotericin B oral suspension in cancer patients with mucositis. *Oral Oncol.* **2002**, *38*, 337–342.
34. A.C. Pasqualotto; Nedel, W. L.; Machado, T. S.; Severo, L. C. Risk factors and outcome for nosocomial breakthrough candidaemia. *J. Infect.* **2006**, *52*, 216–222.
35. Pappas, P. G.; Kauffman, C. A.; Andes, D. R.; Clancy, C. J.; Marr, K. A.; Ostrosky-Zeichner, L.; Reboli, A. C.; Schuster, M. G.; Vazquez, J. A.; Walsh, T. J.; Zaoutis, T. E.; Sobel, J. D. Clinical Practice Guideline for the Management of Candidiasis: 2016 Update by the Infectious Diseases Society of America. *Clin. Infect. Dis.* **2015**, *62*, e1–e50, doi:10.1093/cid/civ933.
36. Synytsya, A.; Novák, M. Structural diversity of fungal glucans. *Carbohydr Polym.* **2013**, *92*, 792–809.
37. Mitchell, K. F.; Zarnowski, R.; Andes, D. R. Fungal Super Glue: The Biofilm Matrix and Its Composition, Assembly, and Functions. *PLOS Pathog.* **2016**, *12*, e1005828, doi:10.1371/journal.ppat.1005828.
38. Zarnowski, R.; Westler, W. M.; Lacmbouh, G. A.; Marita, J. M.; Bothe, J. R.; Bernhardt, J.; Sahraoui, A. L. H.; Fontainei, J.; Sanchez, H.; Hatfeld, R. D.; Ntambi, J. M.; Nett, J. E.; Mitchell, A. P.; Andes, D. R. Novel entries in a fungal biofilm matrix encyclopedia. *MBio* **2014**, *5*, 1–13, doi:10.1128/mBio.01333-14.
39. Johansson, L.; Virkki, L.; Anttila, H.; Esselström, H.; Tuomainen, P.; Sontag-Strohm, T. Hydrolysis of βglucan. *Food Chem.* **2006**, *97*, 71–79, doi:10.1016/j.foodchem.2005.03.031.
40. Rodrigues, C. F.; Silva, S.; Azeredo, J.; Henriques, M.; Henrique, M.; Henriques, M. Detection and Quantification of Fluconazole Within Candida glabrata Biofilms. *Mycopathologia* **2015**, *179*, 391–5, doi:10.1007/s11046-015-9862-9.
41. Hemilä, H.; Louhiala, P. Vitamin C may affect lung infections. *J. R. Soc. Med.* **2007**, *100*, 495–498, doi:10.1258/jrsm.100.11.495.
42. Hemila H. Do vitamins C and E affect respiratory infections?, University of Helsinki., 2007.
43. Sezikli, M.; Çetinkaya, Z. A.; Sezikli, H.; Güzelbulut, F.; Tiftikçi, A.; Tüzün Ince, A.; Gökden, Y.; Yaşar, B.; Atalay, S.; Övünç Kurdaş, O. Oxidative stress in helicobacter pylori infection: Does supplementation with Vitamins C and e increase the eradication rate? *Helicobacter* **2009**, *14*, 280–285, doi:10.1111/j.1523-5378.2009.00686.x.
44. Sezikli, M.; Çetinkaya, Z. A.; Güzelbulut, F.; Yeşil, A.; Coşgun, S.; Kurdaş, O. Ö. Supplementing Vitamins C and e to standard triple therapy for the eradication of Helicobacter pylori. *J. Clin. Pharm. Ther.* **2012**, *37*, 282–285, doi:10.1111/j.1365-2710.2011.01286.x.
45. Ma, J. L.; Zhang, L.; Brown, L. M.; Li, J. Y.; Shen, L.; Pan, K. F.; Liu, W. D.; Hu, Y.; Han, Z. X.; Crystal-Mansour, S.; Pee, D.; Blot, W. J.; Fraumeni, J. F.; You, W. C.; Gail, M. H. Fifteen-year effects of helicobacter pylori, garlic, and vitamin treatments on gastric cancer incidence and mortality. *J. Natl. Cancer Inst.* **2012**, *104*, 488–492, doi:10.1093/jnci/djs003.
46. Sadeghpour, A.; Alizadehasl, A.; Kyavar, M.; Sadeghi, T.; Moludi, J.; Gholizadeh, F.; Totonchi, Z.; Ghadrdoost, B. Impact of Vitamin C Supplementation on Post-Cardiac Surgery ICU and Hospital Length of Stay. *Anesthesiol. Pain Med.* **2015**, *5*, 1–5, doi:10.5812/aapm.25337.
47. Silva, S.; Henriques; Martins, A.; Oliveira, R.; Williams, D.; Azeredo, J. Biofilms of non-Candida albicans Candida species: quantification, structure and matrix composition. *Med Mycol.* **2009**, *47*, 681–689.
48. Markiewicz, M.; Dzierzak-Mietla, M.; Frankiewicz, A.; Zielinska, P.; Koclega, A.; Kruszelnicka, M.; Kyrcz-Krzemien, S. Treating oral mucositis with a supersaturated calcium phosphate rinse: Comparison with control in patients undergoing allogeneic hematopoietic stem cell transplantation. *Support. Care Cancer* **2012**, *20*, 2223–2229, doi:10.1007/s00520-012-1489-5.
49. Rao, N. G.; Han, G.; Greene, J. N.; Tanvetyanon, T.; Kish, J. A.; De Conti, R. C.; Chuong, M. D.; Shridhar, R.; Biagioli, M. C.; Caudell, J. J.; Trotti, A. M. Effect of prophylactic fluconazole on oral mucositis and candidiasis during radiation therapy for head-and-neck cancer. *Pract. Radiat. Oncol.* **2013**, *3*, 229–233, doi:10.1016/j.prro.2012.05.008.
50. Faure, A. M.; Sánchez-Ferrer, A.; Zabara, A.; Andersen, M. L.; Nyström, L. Modulating the structural properties of βd-glucan degradation products by alternative reaction pathways. *Carbohydr. Polym.* **2014**, *99*, 679–686, doi:10.1016/j.carbpol.2013.08.022.
51. Kivelä, R.; Gates, F.; Sontag-Strohm, T. Degradation of cereal beta-glucan by ascorbic acid induced oxygen radicals. *J. Cereal Sci.* **2009**, *49*, 1–3, doi:10.1016/j.jcs.2008.09.003.
52. Xu, H.; Nobile, C.; Dongari-Bagtzoglou, A. Glucanase Induces Filamentation of the Fungal Pathogen Candida albicans. *PLoS One* **2013**, *8*, 1–7, doi:10.1371/journal.pone.0063736.
53. Al-Fattani, M. A.; Douglas, L. J. Biofilm matrix of Candida albicans and Candida tropicalis: Chemical composition and role in drug resistance. *J. Med. Microbiol.* **2006**, *55*, 999–1008, doi:10.1099/jmm.0.46569-0.
54. Kivelä, R.; Nyström, L.; Salovaara, H.; Sontag-Strohm, T. Role of oxidative cleavage and acid hydrolysis of oat

beta-glucan in modelled beverage conditions. *J. Cereal Sci.* **2009**, *50*, 190–197, doi:10.1016/j.jcs.2009.04.012.
55. Kivelä, R.; Sontag-Strohm, T.; Loponen, J.; Tuomainen, P.; Nyström, L. Oxidative and radical mediated cleavage of beta-glucan in thermal treatments. *Carbohydr. Polym.* **2001**, *85*, 645–652.
56. Faure, A. M.; Andersen, M. L.; Nyström, L. Ascorbic acid induced degradation of beta-glucan: Hydroxyl radicals as intermediates studied by spin trapping and electron spin resonance spectroscopy. *Carbohydr. Polym.* **2012**, *87*, 2160–2168, doi:10.1016/j.carbpol.2011.10.045.
57. Shan, N. T.; Nasir, M.; Desa, M.; Sandai, D.; Pei, C. P.; Lung, L. T. T. Growth, biofilm formation, antifungal susceptibility and oxidative stress resistance of Candida glabrata are affected by different glucose concentrations. *Infect Genet Evol.* **2015**, *S1567-1348*, 00371–8.
58. von Sonntag C. *Advances in Carbohydrate chemistry and biochemistry*; Academic Press: New York, 1980;
59. Moura, F. A. de; Pereira, J. M.; Silva, D. O. da; Zavareze, E. D. R.; Moreira, A. da S.; Helbig, E.; Al., E. Effects of oxidative treatment on the physicochemical, rheological and functional properties of oat βglucan. *Food Chem.* **2011**, *128*, 982–987.
60. Faure, A. M.; Münger, L. H.; Nyström, L. Potential inhibitors of the ascorbate-induced beta-glucan degradation. *Food Chem.* **2012**, *134*, 55–63.
61. DuBois, M.; Gilles, K.; Hamilton, J.; Rebers, P.; Smith, F. Colorimetric Method for Determination of Sugars and Related Substances. *Anal. Chem.* **1956**, *28*, 350–356, doi:10.1021/ac60111a017.
62. Freimund, S.; Janett, S.; Arrigoni, E.; Amadò, R. Optimised quantification method for yeast-derived 1,3-βD-glucan and αD-mannan. *Eur. Food Res. Technol.* **2005**, *220*, 101–105, doi:10.1007/s00217-004-1008-0.
63. Danielson, M. E.; Dauth, R.; Elmasry, N. A.; Langeslay, R. R.; Magee, A. S.; Will, P. M. Enzymatic Method To Measure β1,3-β1,6-Glucan Content in Extracts and Formulated Products (GEM Assay). *J. Agric. Food Chem.* **2010**, *58*, 10305–10308, doi:10.1021/jf102003m.
64. Rodrigues, C. F.; Silva, S.; Azeredo, J.; Henriques, M. Detection and Quantification of Fluconazole Within Candida glabrata Biofilms. *Mycopathologia* **2015**, *179*, doi:10.1007/s11046-015-9862-9.

IV

Chapter IV

Candida glabrata biofilms response to polyenes and in combination with new azoles

The work presented in this chapter was adapted from:

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Rodrigues, C.F.; Henriques, M. Liposomal and Deoxycholate Amphotericin B Formulations: Effectiveness against Biofilm Infections of *Candida* spp. *Pathogens*, **2017**;*6*(62). doi: 10.3390/pathogens6040062.

Rodrigues, C.F.; Alves, D.; Henriques, M. Association of posaconazole and amphotericin B in the treatment of biofilms of *Candida glabrata.* Submitted (**2018**).

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IV.1
Candida glabrata recurrent infections: biofilms formation during Amphotericin B treatment

ABSTRACT

Candida spp. are responsible for recurrent human infections, mostly in immunocompromised patients, due to their high vulnerability. *Candida glabrata* has a major role in systemic candidiasis and Amphotericin B (AmB), a hospital environment exclusive polyene, is frequently used to treat this disease. Lately, however, clinical evidences of *Candida* spp. recurrent infections during these treatments are being described, probably due to biofilms (re)formation during this therapy.

Thus, this work aims at inferring if *C. glabrata* biofilms are still being formed during AmB treatment. For that, *C. glabrata* biofilms were formed in the presence of AmB and analysed by dry weight. Matrix composition was analysed quantifying carbohydrates and, specifically, β-1,3-glucans.

Results demonstrated that, although in a lesser extent, *C. glabrata* is able to develop biofilms in the presence of AmB, with a thick extracellular matrix, with an increase on carbohydrates, especially β-1,3-glucans. Therefore, it is confirmed that complex biofilms of *C. glabrata* can be formed during an AmB treatment.

Keywords: amphotericin B, biofilm cells, biofilms, *Candida glabrata*, matrix composition.

IV.1.1. Introduction

Infections caused by *Candida* spp. are a problem of increasing clinical significance, which can result in septicemia or systemic infections, with high morbility and mortality [1]. *Candida* spp.can be found in the gastrointestinal, genital and urinary tract of healthy individuals as innocuous commensals. However, in immunocompromised and/or hospitalized hosts they are able to cause superficial infections, which may lead to invasive and very critical complications. In addition, *Candida* spp. possess high capability to adapt to different niches and *to* invade several epithelia, resulting in septicemia [1,2]. Yet, Amphotericin B, a fungicidal polyene, has shown efficacy activity against many *Candida* spp. [2–6].

Among the several *Candida* spp., *Candida glabrata* is the one of the most prevalent pathogenic fungal species in humans, after *Candida albicans* [7,8]. Besides being very resistant to antifungal agents [1–3], and although lacking capability to produce hyphae, *C. glabrata* possess a number of virulence factors, turning it very aggressive. Among these are included the adhesion to host cells or to medical devices (e.g catheters, algaliation devices) composed by silicone, latex or polyurethane for example, the secretion of hydrolytic enzymes as phospholipases and haemolysins, and the biofilm formation capacity [9,10]. These biofilms can be formed on the host mucosa and/or on surfaces of medical indwelling devices and are composed by yeast cells embedded in a complex polymeric structure, which makes them much more resistant to treatments than original planktonic cells [9,11–13]. Moreover, it was already shown that even after standard treatment of infections caused by biofilms associated to medical devices, some patients still undergo recurrent candidiasis [14].

Therefore, the main goal of this work was to understand why *C. glabrata* infections are still recurrent even during patient's treatment with AmB, by evaluation of its capacity to form biofilms.

IV. 2.1. Material and Methods

VI.2.1.1 Organisms

Two clinical isolates of *C. glabrata* (*C. glabrata* 534784 from vaginal site and *C. glabrata* 562123 from urine) and one reference strain (*C. glabrata* ATCC2001) were used in this work. The identity of all isolates was confirmed using CHROMagar™ *Candida* (CHROMagar™, Paris, France) and by PCR-based sequencing using specific primers (*ITS1* and *ITS4*) against the 5.8s subunit gene reference. Genomic DNA was extracted following previously described procedures [15]. The PCRs

products were sequenced using the ABI-PRISM Big Dye terminator cycle sequencing kit (Perkin Elmer, Applied Biosystems, Warrington, UK).

VI.2.1.2Growth conditions

For each experiment, strains were subcultured on Sabouraud dextrose agar (SDA) (Merck, Darmstadt, Germany) for 24 h at 37°C. Cells were then inoculated in Sabouraud dextrose broth (SDB) (Merck, Darmstadt, Germany) and incubated for 18 h at 37°C under agitation at 120 rotations per minute ($rpm.min^{-1}$). After incubation, the cells were harvested by centrifugation at 3000 *g* for 10 min at 4°C and washed twice with Phosphate Buffered Saline (PBS 0.1 *M*, pH=7.5). Pellets were then suspended in SDB and the cellular density was adjusted to $1x10^5$ cells/mL using a Neubauer counting chamber [13].

VI.2.1.3Amphotericin B

AmB (Sigma-Aldrich, St Louis, USA), was prepared at 1 mg/L and 2 mg/L, from a stock of 1000 mg/L, diluted in dimethyl-sulfoxyde.

VI.2.1.4Biofilm formation and analysis

Standardized cell suspensions were placed into selected wells of 24-wells polystyrene microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium) plus 250 µL of each duplicated concentration of antifungal to test (1mg/L and 2 mg/L, final concentrations). As negative control SDB without cells and antifungal agent was used. As positive control, cells suspensions were tested without antifungal agent. After 24h, 250 µL of SDB medium was removed and an equal volume of fresh SDB plus the respective antifungal concentration agent were added. Dry weight was analyzed to evaluate AmB effect on biofilm production [13].

In order to examine the structure of biofilms, after formation in the presence or absence of AmB, they were observed by scanning electron microscopy. For that, biofilms formed as described above were dehydrated with ethanol (using 70% ethanol for 10 min, 95% ethanol for 10 min and 100% ethanol for 20 min) and air dried for 20 min. Samples were kept in a desiccator until the base of the wells was removed for analysis. Prior to observation, the base of the wells was mounted onto aluminum stubs, sputter coated with gold and observed with an S-360 scanning electron microscope (Leo, Cambridge, USA).

VI.2.1.4.1 Total carbohydrate content

Total carbohydrate content of the biofilm matrix was estimated according to the procedure of Dubois et al [16], using glucose as standard. The values were normalized per g of dry weight biofilm and the values presented as mg of carbohydrate/g of dry weight of biofilm.

VI.2.1.4.2 β-1,3-glucans concentrations

β-1,3-glucans concentrations were determined using Glucatell® kit (Cape Cod®, East Falmouth, USA). The values were normalized by mg of β-1,3-glucans per g dry weight of biofilm.

VI.2.1.5 Statistical Analysis

All the experiments were performed in triplicate and in three independent assays. Results were compared using a one-way ANOVA, Dunnett's post hoc multiple comparisons tests, using GraphPad™ Prism 5™ (CA, USA) software. All tests were performed with a confidence level of 95%.

VI.3.1. Results and Discussion

The ability of *C. glabrata* to form biofilms in the presence of AmB was determined by dry weight (Table IV.1.1).

Table IV.1.1. Effect of amphotericin B on *Candida glabrata* biofilm formation by dry weight determination (*** P<0.0001)

C. glabrata **strain**	**[AmB] mg/L**	**Dry weight of biofilm mg ± SD (*P* value)**
ATCC 2001	0	20 ± 0.5
	1	12 ± 0.2 ***
	2	10 ± 0.5 ***
562123	0	20 ± 0.4
	1	10 ± 0.0 ***
	2	0.8 ± 0.2 ***
534784	0	30 ± 0.2
	1	13 ± 0.5 ***
	2	16 ± 0.6 ***

C. glabrata 534784 showed to have higher capacity to produce biofilm, comparing to the other two strains (Table IV.1.1), which can also be noticed in the SEM images (Figure IV.1.1I, IV.1II and IV.1III). Importantly, it can be remarked that biofilms are, in fact, still being developed by the three strains in the presence of AmB to a lesser extent and they even present a considerable amount of

biomass, except for *C. glabrata* 562123, in the presence of the highest concentration of AmB (Table IV.1.1). It should also be highlighted that this corresponds to usual therapeutic doses.

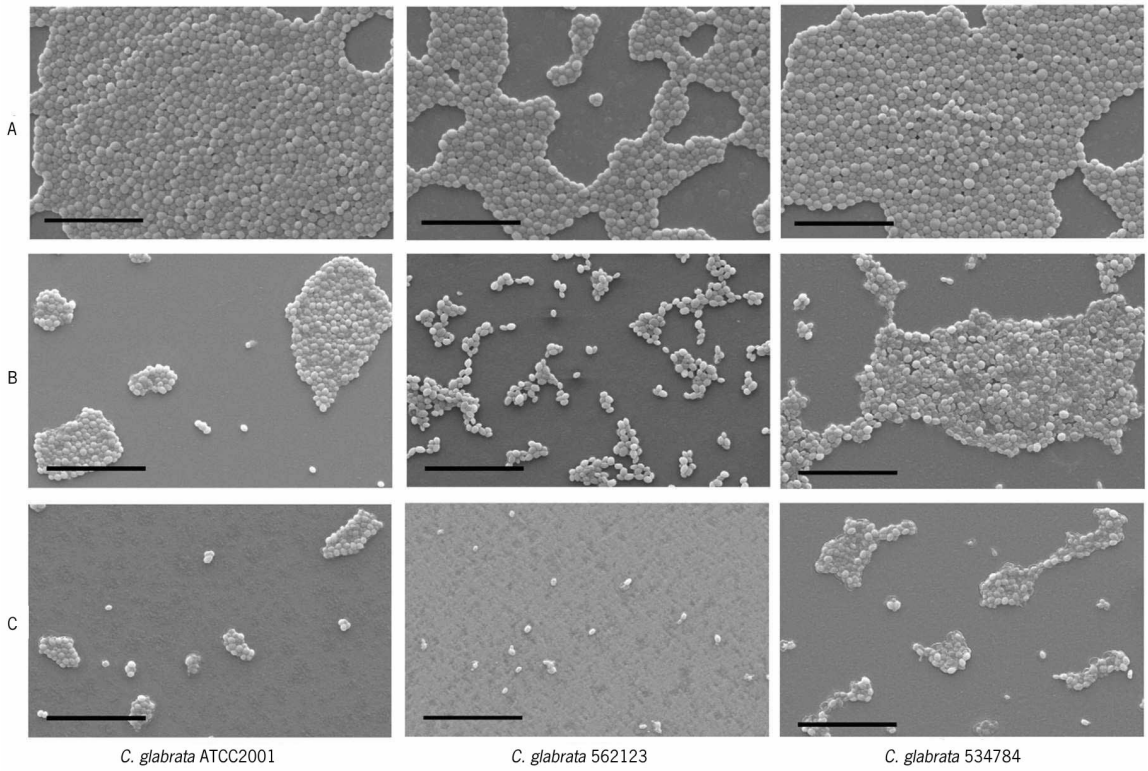


Figure IV.1.1. SEM images of *Candida glabrata* biofilms. Magnification: 1000x. A – Biofilm grown without AmB; B – Biofilm grown with 1 mg/L of AmB; C – Biofilm grown with 2 mg/L of AmB. (Measure bar = 20 µm).

SEM images (Figure IV.1.1) confirmed that, although *C. glabrata* biofilm formation is reduced in the presence of AmB, an organized structure can still be observed (Figure IV.1.1B and IV.1.1C). This allowed the consideration that AmB could cause structural modifications on *C. glabrata* biofilm, with the presence of a compact matrix. In fact, one of the major contributions to *C. glabrata* virulence is its versatility in being adapted to a variety of different habitats and the formation of biofilms [8,11,14,17]. There is a general consensus that the biofilm matrix acts as a barrier to the diffusion of antimicrobial agents, thereby limiting the access of the antimicrobial to organisms at the lower layers of the biofilm [8,11,14,17]. However, little is known about the effect of the antifungal agent on the matrix production and composition.

Thus, posteriorly, the evaluation of the matrix composition (Figure IV.1.2) confirmed the presence of rich and mature biofilm matrices in the presence of AmB. It was possible to verify an increase

in the amount of carbohydrates on the matrix on the biofilms formed in the presence of AmB (Figure IV.1.2), especially of β-1,3-glucans (Table IV.1.2).

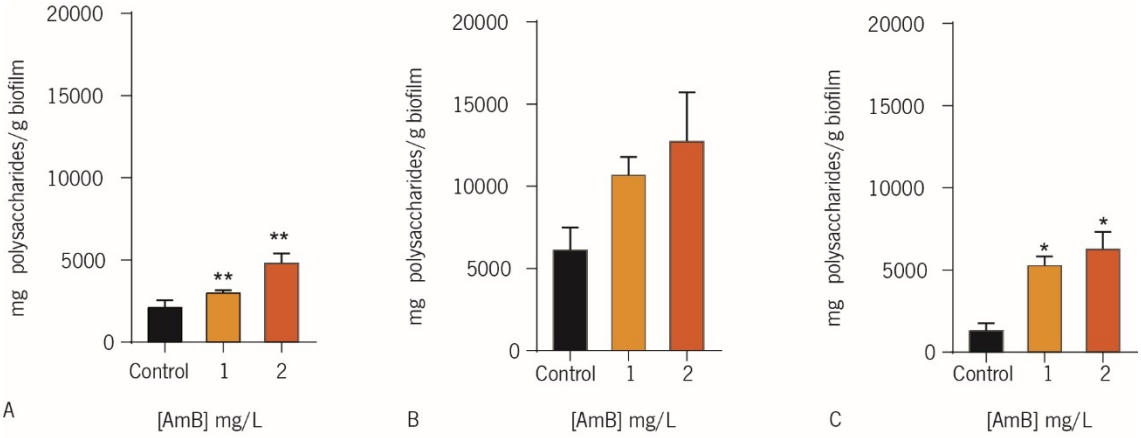


Figure IV.1.2. Polysaccharides content on *Candida glabrata* biofilms matrices with and without Amphotericin B (* P<0.05; ** P<0.01) (A – *C. glabrata* ATCC2001; B - *C. glabrata* 562123; C - *C. glabrata 534784*).

β-1,3- glucans are polymers found in cell wall of *Candida* spp. and in biofilm matrix and are related to the antifungal resistance, making difficult to the drugs to diffuse through the biofilm matrices [18–20]. In fact, the increase of these compounds in biofilm matrix is very notorious even when there is a significant reduction of the total biofilm (Table IV.1.1 and Figure IV.1.1). Meaning that, even with a reduction on the number of biofilm cells, the increase of biofilm matrix is notorious and maybe associated to the stress caused by the antifungal agent.

Table IV.1.2. β-1,3-glucans quantity in *Candida glabrata* biofilms matrices, in presence and absence of amphotericin B (*** *P*<0.0001)

C. glabrata **strain**	**[AmB] mg/L**	**pg β-1,3-glucans per g of dry weight of biofilm (*P* value)**
ATCC2001	0	$5.15x10^{-5}$
	1	$5.80x10^{-5}$ ***
	2	$3.29x10^{-5}$ ***
562123	0	$1.79x10^{-5}$
	1	$5.43x10^{-5}$ ***
	2	$6.78x10^{-5}$ ***
534784	0	$3.13x10^{-5}$
	1	$6.95x10^{-5}$ ***
	2	$4.63x10^{-5}$ ***

It can be conjectured that AmB might be triggering the production and secretion of carbohydrates, which may be related with an attempt to make a physical protection for the cells, against the

antifungal aggression, as it has already been demonstrated for *C. albicans* [18]. In fact, glucan enzymes responsible for the production of these carbohydrates seem to play a biofilm specific role in facilitating the delivery and organization of mature biofilm matrices, being decisive for delivery of β-1,3-glucans to the biofilm matrices and for accumulation of mature matrices biomass[18]. Concluding, it was confirmed that *C. glabrata* can develop biofilms in the presence of therapeutic concentrations of AmB, due to the high carbohydrate and β-1,3-glucans concentrations quantified on the biofilm matrices, highlighting the capacity of *Candida* spp. cells to rapidly overcome outside aggressions and realizing why patients undergoing AmB treatment still manifest resilient *Candida* spp. infections. Besides, this high carbohydrate content, specially β-1,3-glucans can also justify the biofilm cells high resistance to antifungal treatments that have been typical of *C. glabrata*.

References

1. Lass-Flörl, C. The changing face of epidemiology of invasive fungal disease in Europe. *Mycoses* **2009**, *52*, 197–205.
2. Ellis, D. Amphotericin B: spectrum and resistance. *J. Antimicrob. Chemother.* **2002**, *49 Suppl 1*, 7–10.
3. Krogh-Madsen, M.; Arendrup, M. C. M. C.; Heslet, L.; Knudsen, J. D. J. D. Amphotericin B and caspofungin resistance in Candida glabrata isolates recovered from a critically ill patient. *Clin. Infect. Dis.* **2006**, *42*, 938–944., doi:10.1086/500939.
4. Laniado-Laborin, R.; Cabrales-Vargas, M. Amphotericin B: side effects and toxicity. *Rev Iberoam Micol* **2009**, *26*, 223–7.
5. Baginski, M.; Czub, J. Amphotericin B and its new derivatives-mode of action. *Curr Drug Metab* **2009**, *10*, 459–69.
6. Vandeputte, P.; Tronchin, G.; Larcher, G.; Ernoult, E.; Bergès, T.; Chabasse, D.; Bouchara, J.-P. A nonsense mutation in the ERG6 gene leads to reduced susceptibility to polyenes in a clinical isolate of Candida glabrata. *Antimicrob. Agents Chemother.* **2008**, *52*, 3701–9, doi:10.1128/AAC.00423-08.
7. West, L.; Lowman, D.; Mora-Montes, H.; Grubb, S.; Murdoch, C.; Thornhill, M.; Gow, N.; Williams, D.; Haynes, K. Differential virulence of Candida glabrata glycosylation mutants. *Biol Chem.* **2013**, *288*, 22006–18.
8. Angoulvant, A.; Guitard, J.; Hennequin, C. Old and new pathogenic *Nakaseomyces* species: epidemiology, biology, identification, pathogenicity and antifungal resistance. *FEMS Yeast Res.* **2015**, *16*, fov114, doi:10.1093/femsyr/fov114.
9. Rodrigues, C. F.; Silva, S.; Henriques, M. Candida glabrata: A review of its features and resistance. *Eur. J. Clin. Microbiol. Infect. Dis.* **2014**, *33*, 673–688, doi:10.1007/s10096-013-2009-3.
10. Sánchez-Vargas, L. O.; Estrada-Barraza, D.; Pozos-Guillen, A. J.; Rivas-Caceres, R. Biofilm formation by oral clinical isolates of Candida species. *Arch. Oral Biol.* **2013**, *58*, 1318–26, doi:10.1016/j.archoralbio.2013.06.006.
11. Sardi, J. C. O.; Scorzoni, L.; Bernardi, T.; Fusco-Almeida, A. M.; Mendes Giannini, M. J. S. Candida species: Current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. *J. Med. Microbiol.* **2013**, *62*, 10–24, doi:10.1099/jmm.0.045054-0.
12. d'Enfert, C.; Janbon, G. Biofilm formation in Candida glabrata: What have we learnt from functional genomics approaches? *FEMS Yeast Res.* **2016**, *16*, fov111, doi:10.1093/femsyr/fov111.
13. Rodrigues, C. F.; Silva, S.; Azeredo, J.; Henriques, M.; Henrique, M.; Henriques, M. Detection and Quantification of Fluconazole Within Candida glabrata Biofilms. *Mycopathologia* **2015**, *179*, 391–5, doi:10.1007/s11046-015-9862-9.
14. Mishra, N.; Prasad, T.; Sharma, N.; Payasi, A.; Prasad, R.; Gupta, D.; Singh, R. Pathogenicity and drug resistance in Candida albicans and other yeast species. *Acta Microbiol Immunol Hung.* **2007**, *54*, 201–235.
15. Williams, D. W.; Wilson, M. J.; Lewis, M. A. O.; Potts, A. J. C. Identification of Candida Species by PCR and Restriction Fragment Length Polymorphism Analysis of Intergenic Spacer Regions of Ribosomal DNA. *J. Clin. Microbiol.* **1995**, *33*, 2476–2479.
16. DuBois, M.; Gilles, K.; Hamilton, J.; Rebers, P.; Smith, F. Colorimetric Method for Determination of Sugars and Related Substances. *Anal. Chem.* **1956**, *28*, 350–356, doi:10.1021/ac60111a017.

17. Donlan, R.; Costerton, J. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.* **2002**, *15*, 167–193.
18. Taff, H. T.; Nett, J. E.; Zarnowski, R.; Ross, K. M.; Sanchez, H.; Cain, M. T.; Hamaker, J.; Mitchell, A. P.; Andes, D. R. A Candida biofilm-induced pathway for matrix glucan delivery: implications for drug resistance. *PLoS Pathog.* **2012**, *8*, e1002848, doi:10.1371/journal.ppat.1002848.
19. Zarnowski, R.; Westler, W. M.; Lacmbouh, G. A.; Marita, J. M.; Bothe, J. R.; Bernhardt, J.; Sahraoui, A. L. H.; Fontainei, J.; Sanchez, H.; Hatfeld, R. D.; Ntambi, J. M.; Nett, J. E.; Mitchell, A. P.; Andes, D. R. Novel entries in a fungal biofilm matrix encyclopedia. *MBio* **2014**, *5*, 1–13, doi:10.1128/mBio.01333-14.
20. Lewis, R. E.; Viale, P.; Kontoyiannis, D. P. The potential impact of antifungal drug resistance mechanisms on the host immune response to Candida. *Virulence* **2012**, *3*, 368–76, doi:10.4161/viru.20746.

IV.2

Liposomal and deoxycholate Amphotericin B formulations: effectiveness against biofilms infections of *Candida* spp.

ABSTRACT

Background: candidiasis is the primary fungal infection encountered in patients undergoing prolonged hospitalization and the fourth leading cause of nosocomial bloodstream infections. One of the most important *Candida* spp. virulence factors is the ability to form biofilms, which are extremely refractory to antimicrobial therapy and very difficult to treat with the traditional antifungal therapies. Since the choices regarding the treatment or the prophylaxis are repeatedly taken without considering the possibility of a *Candida* spp. biofilm-related infection, it is important to assess the effectiveness of the actual drugs and which formulations have the best performance in these infections-biofilm forms.

Material and Methods: 24-h-biofilms of four *Candida* spp. and their response to two amphotericin B (AmB) pharmaceutical formulations were evaluated: liposomal and deoxycholate.

Results: generally, *Candida glabrata* was the less susceptible yeast species to both AmBs. MBECs revealed that it is therapeutically more appealing using AmB-L than AmB-Deox for all *Candida* spp. biofilms, since none of the determined concentrations of AmB-L reached 10% of the maximum daily dose, but both formulations showed a very good capacity in biomass reduction.

Conclusion: the liposomal formulation presents better performance in eradication of the biomass and biofilm cells, for all the species, in comparison with the deoxycholate formulation.

Keywords: Amphotericin B, Liposomal, Deoxycholate, *Candida* spp., Biofilms, Drug resistance.

IV.1.2. Introduction

Infections caused by *Candida* spp. have increased significantly in the past 30 years, becoming a substantial cause of morbidity and mortality. This is particularly critical in immunologically compromised individuals, in patients submitted to continuous treatment with broad-spectrum antibiotics or to invasive procedures and with medical implanted devices, which can cause both superficial and systemic infections [1,2]. Although *Candida albicans* is, generally, the most frequently isolated species, there has also been a noteworthy upsurge in the frequency of non-*Candida albicans* species, such as *Candida glabrata, Candida parapsilosis* and *Candida tropicalis* [3]. *Candida* spp. pathogenicity is mediated by a number of virulence factors, including the ability to adhere to medical devices and to host cells, often leading to the formation of biofilms [4]. Biofilms are biological communities with an extraordinary degree of organization, in which microorganisms form structured, coordinated, and functional communities, embedded in a self-created extracellular matrix [1,2,5]. The formation of *Candida* spp. biofilms raises significant clinical issues because of an additional increase in antifungal drug's resistance, as well as, evasion of host immune defences. Furthermore, biofilm development on medical devices can cause the failure of the device and may turn into a source for future infections [6–8].

Polyenes, are among the most effective drugs for the treatment of systemic *Candida* spp. infections, specifically AmB [9,10]. AmB binds to the ergosterol of the fungal cell membrane establishing transmembrane aggregates pores which causes membrane depolarization with subsequent increase in membrane permeability to monovalent protons and cations. This allows the passage of intracellular molecules to the external environment, initiating an osmotic imbalance and finally cell death [11–13]. This drug, produced by *Streptomyces nodosus,* is part of the macrolides class, characterized by a macrocyclic ring lactone, with a hydrophobic and one hydrophilic domain which gives an amphipathic characteristic that confers low solubility in aqueous solutions at physiological pH. Hence, AmB has low bioavailability via oral administration, since it is a highly hydrophobic weak base with a low aqueous solubility of approximately 1 ng/mL at pH 7 [14,15]. This fact led to the production of a complex with another agent to enable its clinical use: sodium deoxycholate. The possible administration routes are intravenous, intra-articular, intravesical, intrathecal, in injuries and applied in surgical sites [12,13,16] .

AmB deoxycholate (AmB-Deox) has been used during the past years due to the rise in the number of immunosuppressed patients suffering invasive fungal infections, but has been related to a high rate of side effects, particularly renal toxicity [13,17]. Therefore, other formulations were

developed: a lipid formulation (liposomal, AmBisome®, Gilead Sciences, Inc.), a lipid complex (Abelcet®, Sigma Tau Pharmaceuticals, Inc.), and a colloidal suspension (Amphocil®, Penn Pharmaceuticals, Ltd.), which share the same antifungal spectrum but differ in efficacy and toxicity [13,17]. The liposomal formulation (AmB-L) is constituted by 50-100 μm spheres and is composed of hydrogenated phosphatidylcholine soy, 25% of cholesterol, sterically attached to distearylphosphatidyl glycerol (DSPG) and AmB. Every AmB molecule within the liposome is complexed with DSPG and cholesterol, which allows it to escape initial clearance of the sarcoplasmatic reticulum, but once captured, the concentrations in liver and spleen increase as it decreases in the plasma. Consequently, it is guaranteed that there are no AmB-L residues in blood, reaching the highest serum concentrations [13,18–22].

The first cases of resistance to polyenes' treatment are related with the increase of systemic infections, many of them with primary or intrinsic resistance to AmB and consistently associated with high mortality rate [23–25]. These cases have been increasing, but fortunately the studies still recognize the high effectiveness of AmB-Deox and AmB-L on planktonic cells or in the prevention of the biofilm formation of *Candida* spp. [26–31]. On the other side, fewer researchers have performed studies specifically on the activity of the two formulations on matured biofilms [32,33]. Since these communities are known to be responsible for the most aggressive systemic infections [2], the aim of this study was to evaluate the efficacy of the AmB liposomal formulation (AmbiSome®) (comparing to the original, deoxycholate (Fungizone®) in eliminating the cells derived from matured biofilms of the four most common *Candida* spp. found in hospitals: *Candida albicans*, *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*.

IV.2.2 Material and Methods

VI.2.2.1 Organisms and growth conditions

Four reference species of *Candida* spp., were used in this study: *C. albicans* SC5314 and three from the American Type Culture Collection (ATCC), *C. glabrata* ATCC2001, *C. parapsilosis* ATCC22019 and *C. tropicalis* ATCC750. For each experiment, yeasts were subcultured on Sabouraud dextrose agar (SDA) (Merck, Darmstadt, Germany) for 24 h at 37°C. Cells were then inoculated in Sabouraud dextrose broth (SDB) (Merck, Darmstadt, Germany) and incubated for 18 h at 37°C under agitation at 120 rpm. After incubation, the cells were harvested by centrifugation at 3000 *g* for 10 min at 4°C and washed twice with phosphate buffered saline (PBS 0.1 *M*,

pH=7.5). Pellets were then suspended in RPMI-1640 (Sigma-Aldrich, USA) and the cellular density was adjusted to $1x10^5$ cells/mL, using a Neubauer counting chamber.

VI.2.2.2Antifungal drugs

AmB-Deox was purchased from Sigma® (Sigma-Aldrich, Roswell Park) and AmB-L was supplied by Gilead Sciences, Inc. (Foster City, CA). Aliquots of 2000 mg/L were prepared using dimethyl-sulfoxide (DMSO) for AmB-Deox, and according to the indications of the manufacturer for AmB-L.

VI.2.2.3Antifungal susceptibility tests

The antifungal susceptibility tests, for both formulations of AmB, were determined using the microdilution method, in accordance to the European Committee on Antimicrobial Susceptibility Testing guidelines (EUCAST) [34,35].

VI.2.2.4Planktonic Susceptibility Evaluation

VI.2.2.4.1 Minimum Inhibitory Concentrations (MICs)

The AmB MIC is the lowest concentration, recorded in mg/L, of the drug that inhibits the growth of the yeasts to a predefined degree (e.g. 90% in the case of polyenes) [35]. The MIC informs about the susceptibility or resistance of the *Candida* spp. to the AmB formulations [34,36],

The AmB concentrations tested were prepared in RPMI-1640 (pH=7, Sigma-Aldrich, Roswell Park). The inoculum was prepared by suspending five distinct colonies, ≥1 mm diameter from 24 h cultures, in at least 3 mL of sterile distilled water. Then, the inoculum was suspended by vigorous shaking on a vortex mixer for 15 s and the cell density was adjusted to the density of a 0.5 McFarland standard and adding sterile distilled water as required, giving a yeast suspension of 1-$5x10^6$ colony forming units (CFUs) CFU/mL. A working suspension was prepared by a dilution of the standardised suspension in sterile distilled water to yield 1-$5x10^5$ CFU/mL. The 96-well-plate (Orange Scientific, Braine-l'Alleud, Belgium) was prepared with 100 µL of cell suspension and 100 µL of antifungal agent (0.25, 0.5, 1 and 1.5 mg/L, 2x concentrated) and incubated at 37°C, during 18-48 h. Controls without antifungal agents were also performed. Finally, the results were visualized by spectrometry at 530 nm.

VI.2.2.4.2 Minimum Fungicidal Concentration (MFC)

The AmB MFC is the lowest concentration, recorded in mg/L, of the drug that eliminates the planktonic population to at least, 2 Log_{10} CFU per cm^2. For that determination, in addition to the previous step, 20 μL of each cell suspension treated with AmB-Deox and AmB-L was recovered to a new well and serial decimal dilutions in PBS were plated onto SDA. Agar plates were incubated for 24 h at 37 °C, and the total number of CFUs was determined. The results were calculated by Log_{10} CFU per cm^2 (Log_{10} CFUs/cm^2) and presented by mg/L [37].

VI.2.2.5 Biofilm Structure, Susceptibility Evaluation and Biomass Reduction Analysis

VI.2.2.5.1 Scanning Electronic Microscopy (Biofilm structure visualization)

In order to examine the structure of the biofilms of the *Candida* spp., they were observed by scanning electron microscopy. For that, biofilms formed as described above were dehydrated with ethanol (using 70% ethanol for 10 min, 95% ethanol for 10 min and 100% ethanol for 20 min) and air dried for 20 min. Samples were kept in a desiccator until the base of the wells was removed for analysis. Prior to observation, the base of the wells were mounted onto aluminum stubs, sputter coated with gold and observed with an S-360 scanning electron microscope (Leo, Cambridge, USA).

VI.2.2.5.2 Minimum Biofilm Eradication Concentration (MBEC)

The AmB MBEC is the lowest concentration, recorded in mg/L, of the drug able to eliminate the biofilm cells' population to, at least, 2 Log_{10} CFU per cm^2. For that determination, standardized cell suspensions (200 μL) were placed into selected wells of 96-wells polystyrene microtiter plates. RPMI-1640 was used without cells, but with antifungal agent, as a negative control. As positive control cell suspensions were tested without antifungal agent. At 24 h, 100 μL of RPMI-1640 was removed and an equal volume of fresh RPMI-1640 plus the respective antifungal concentration were added (2, 3, 4, 8 mg/L, 2x concentrated). The plates were incubated at 37°C for another 24 h, a total of 48 h at 120 rpm. The number of cultivable cells on biofilms was determined by the enumeration of CFUs. For that, after the period of biofilm formation, all medium was aspired and the biofilms washed once with 200 μL of PBS to remove non-adherent cells. Then, biofilms were scraped from the wells and the suspensions were vigorously vortexed for 2 min to disaggregate cells from the matrix. Serial decimal dilutions in PBS were plated on SDA and incubated for 24 h

at 37ºC. The results were calculated as total of CFUs per unit area (Log_{10} CFUs/cm^2) and presented by mg/L [37].

VI.2.2.5.3 Biofilm total biomass quantification – Crystal Violet Staining

Total biofilm biomass was quantified by crystal violet (CV) staining [38]. After biofilms formation, the medium was aspirated and non-adherent cells removed by washing the biofilms with sterile ultra-pure water. Then, biofilms were fixed with 200 μL methanol, which was removed after 15 min of contact. The microtiter plates were allowed to dry at room temperature, and 200 μL of CV (1% v/v) were added to each well and incubated for 5 min. The wells were then gently washed twice with sterile, ultra-pure water and 200 μL of acetic acid (33% v/v) were added to release and dissolve the stain. The absorbance of the obtained solution was read in triplicate in a microtiter plate reader (Bio-Tek Synergy HT, Izasa, Lisbon, Portugal) at 570 nm. Three negatives were performed using sterile ultra-pure water. The results were presented as percentage of reduction of biomass.

VI.2.2.6 Statistical Analysis

The assays were performed in triplicate and on three separate occasions. Results were compared using two-way ANOVA, Bonferroni's post hoc comparison test, using GraphPad™ Prism 5™ (CA, USA) software. All tests were performed with a confidence level of 95%.

VI.3.2. Results and Discussion

Invasive fungal infections will unquestionably continue to grow with the constant widespread use of immunosuppressive therapy together with broad-spectrum antimycotic therapy, the number of patients at risk in medical care, particularly the rise of severely immunocompromised patients [1,39–41]. Currently, echinocandins are the considered the first choice class of antifungals to treat systemic candidiasis [42,43]. Possibly because of that circumstance, both breakthrough infections and acquired resistance mutations in certain species of *Candida* spp. have been reported (especially in *C. glabrata* infections), which makes the managing of invasive candidiasis a permanent challenge [44,45]. It is, thus, vital to perform a constant assessment if antifungal drugs are still effective, appropriate and clinically safe. AmB, a polyene antifungal drug, binds to the ergosterol and also induces the accumulation of reactive oxygen species, having multiple deleterious and fungicidal effects on fungal cells, which probably explains the low rate of resistance

events associated to this drug [26,46]. Still, AmB has also has the ability to bind to cholesterol, although with lower affinity, which is assumed to be connected to its greatest toxic potential. Due to the high frequency of nephrotoxicity using AmB-Deox [47], pharmaceutical industry has produced formulations with lipids, namely AmB-L [13,48–50]. This work focused in *Candida* spp. infections exclusively resultant from biofilm cells - which are recognized as having a great impact in nosocomial infections but are still poorly understood [51] - and how they responded to two different formulations of AmB.

The results of MIC and MFC can be observed in Table IV.2.1. All four *Candida* spp. were considered susceptible to both AmB formulations, according to the most recent EUCAST breakpoints [34]. Generally, AmB-Deox had a better performance than AmB-L in both tests, showing lower concentrations to eliminate visual growth (MIC) or eliminate, at least, 2 Log_{10} CFU/cm^2 of the initial inoculum (MFC). Regarding to the MIC results, *C. tropicalis* ATCC750 was most tolerant to AmB-Deox (0.5 mg/L, compared to the rest: 0.25mg/L) and *C. albicans* SC5314 the most sensitive to AmB-L (0.5 mg/L, compared to the rest, 1 mg/L). AmB-Deox is a colloidal dispersion with a size of 0.035 nm, counterbalancing with the ≅0.080 nm of size of the spherical AmB-L [13,52]. This fact probably influenced the immediate cellular penetration in the free planktonic cells, enhancing it in the case of the AmB-Deox, when comparing both formulations. In particular, for two of the studied species (*C. glabrata* ATCC2001 and *C. parapsilosis* ATCC22019) AmB-L required a concentration 4 times higher (1 mg/L) than when using AmB-Deox (0.25 mg/L). Similar results have been showed by other authors regarding *C. glabrata* species [53–57]. *C. albicans* SC5314 was the species that demanded lower drug concentrations, for both formulations, responding very well to this polyene, as it has been indicated before [53,58–62]. Concerning the MFC results (Table IV.2.1), the differences between the four species were not so evident. Generally, the concentration that points to the inhibition of growth (MIC) is closer to cell death (MFC) for AmB-L, than for AmB-Deox. Using AmB-L, the concentration used for NCAC species, was 1.5 times higher than MIC and for *C. albicans* SC5314 the value was 3 times higher, showing a higher drug tolerance in this species, which has been demonstrated earlier [63]. With AmB-Deox these variations were more noteworthy. In fact, it seems to have an advantage in the use of AmB-L over AmB-Deox. These variations on *Candida* spp. responses between AmB-Deox and AmB-L can determine the outcome in an infection treatment, especially in biofilms infections, since it is known that the inhibition of growth does not always lead to cell death, but sometimes to cell dormancy or even the appearance of tolerant and persister cells [63–70]. None of the drug formulations demanded high *in vitro* doses

to show efficacy, thus, showing to be good options for the treatment of planktonic *Candida* spp. infections.

Table IV.2.1. Results in MIC and MFC concentrations for both AmB formulations

Species	**MIC (mg/L)**		**MFC (mg/L)**	
	AmB-Deox	**AmB-L**	**AmB-Deox**	**AmB-L**
Candida albicans SC5314	0.25	0.5	1	1.5
Candida glabrata ATCC2001	0.25	1	1	1.5
Candida parapsilosis ATCC22019	0.25	1	1	1.5
Candida tropicalis ATCC750	0.5	1	1	1.5

Next, in order to visualize the appearance of the matured biofilms of the four *Candida* spp., SEM images were performed (Figure IV.2.1).

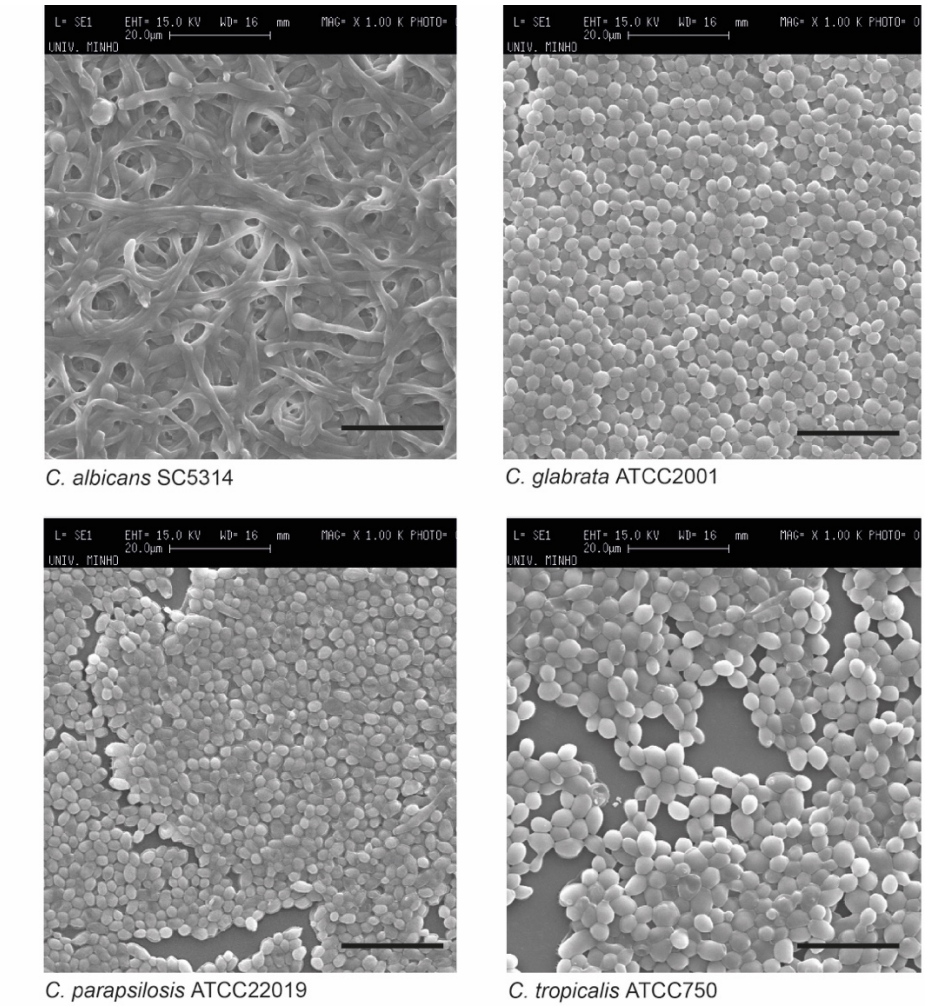


Figure IV.2.1. SEM images of matured biofilms of *Candida albicans* SC5314, *Candida glabrata* ATCC2001, *Candida parapsilosis* ATCC22019 and *Candida tropicalis* ATCC750. Magnification: 1000x (Measure bar = 20 µm).

It was confirmed that all *Candida* spp. could form structured biofilms on the culture conditions applied. Specifically, *C. albicans* SC5314 demonstrated to have a biofilm with high hyphae quantity and entanglement [2,71]. This morphological change (from yeast to hyphae) can influence biofilm formation and stability [2,72]. *C. glabrata* ATCC2001 forms biofilms constituted by yeasts in a long continuous carpet [2,73,74] and *C. parapsilosis* ATCC22019 a continuous biofilm carpet with clumped blastospores [2,75]. Finally, *C. tropicalis* ATCC750 biofilm can be described as chains of cells with high amounts of extracellular material [2,76,77]. These strong biofilms have been related to higher pathogenicity, virulence and resistance, and to difficult the drug diffusion [2,78–81].

In what concerns the main goal of this study, the biofilm treatment and eradication (MBEC, Table IV.2.2), the outcomes changed significantly. Both AmB-L and AmB-Deox required, between 4 and 8 times more drug concentration to eliminate the biofilm cells than the corresponding planktonic cells. Since AmB-Deox and AmB-L have very different permitted daily dose for a systemic candidiasis (AmB-Deox: 1.2 mg/kg/day for an adult of 70 kg, and AmB-L: 6 mg/kg/day [82,83]), in order to ease the comparison of the results, the data were transformed in percentage of maximum permitted daily dose (Table IV.2.2).

Generally, AmB-L showed a better response on the 24-h-pre-formed biofilms. Excepting *C. glabrata* ATCC2001, the three other *Candida* spp. needed 2 mg/L of AmB-Deox to eliminate the biofilm cells which represents approximately 12% of the maximum of the daily dose for this formulation. *C. glabrata* ATCC2001 presented resistant pattern, requiring 4 mg/L (meaning almost 24% of the daily dose), which was not a total surprise, since this species is being reported to have this performance and to have already resistance cases with polyenes [30,84–87]. With AmB-L, the values were slightly more variable. *C. parapsilosis* ATCC22019 was the less resistant species [34], with 2 mg/L (2.38% of the maximum dose), followed by *C. albicans* SC5314 with 3 mg/L (3.57% of the maximum dose) (Table IV.2.2). The MBEC values obtained were higher than reported previously for other *C. albicans* strains [88–90], but were similar for C*. parapsilosis* [91,92]. The differences observed can be due to alterations on the biofilm formation conditions and the fact that the MBEC evaluations methods used not identical. Nonetheless, Prazynka and colleagues [30] had parallel outcomes in pre-formed 24-h-biofilms. *C. glabrata* ATCC2001 and *C. tropicalis* ATCC750 presented a clearer biofilm resistance profile with results ≥8 mg/L and ≥9.52% of the maximum dose (Table IV.2.2). Comparable concentrations for these two species have already been reported by other authors [93–97]. It is noticed, though, that, comparing the percentages of clinical doses,

the required concentrations to eliminate the biofilm are therapeutically more appealing when using AmB-L than AmB-Deox for all *Candida* spp., since none of the determined concentrations of AmB-L even reached 10% of the maximum daily dose (Table IV.2.2). The differences between the percentages of the two AmB formulations, were statistically significant for each species ($P < 0.001$) Finally, regarding the biofilm biomass reduction (Table IV.2.3), it was possible to observe a dependence on species and AmB formulation, but generally, both AmB showed to have good performance, with reductions between 34.64% and 89.58% for AmB-Deox and between 43.78% and 70.72% for AmB-L. *C. tropicalis* ATCC750 and *C. albicans* SC5314 showed a pronounced biofilm reduction with only 0.25 mg/L of AmB-Deox (≅90% and ≅70%, respectively), but the MBEC values demonstrated that the same biofilm cells required 8 times more drug concentration (2 mg/L) to be eliminated.

Table IV.2.2. MBEC values of AmB-Deox and AmB-L and its percentage on the maximum permitted dose used‡

Species	MBEC (mg/L)			
	AmB-Deox	**% of maximum permitted dose#**	**AmB-L**	**% of maximum permitted dose#**
Candida albicans SC5314	2	11.90	3	3.57***
Candida glabrata ATCC2001	4	23.81	≥8	≥9.52***
Candida parapsilosis ATCC22019	2	11.90	2	2.38***
Candida tropicalis ATCC750	2	11.90	≥8	≥9.52***

‡ the difference between the percentages of both AmB formulations are all statistically significant, *** $P < 0.001$;
#It was considered the maximum dose allowed for invasive candidiasis [82,83]: AmB-Deox: (0.6 to) 1.2 mg/kg for an adult of 70 kg; AmB-L: (3 to) 6 mg/kg for an adult of 70 kg

The opposite happened with *C. glabrata* ATCC2001 and *C. parapsilosis* ATCC22019. These two species demanded a concentration near to the MBEC value to eliminate 50% of the biofilm, showing the capacity of producing robust biofilms in abiotic surfaces, as it was described before [98]. Regarding AmB-L, *C. albicans* SC5314 was the species that required less AmB-L concentration to eliminate a higher percentage of its biomass (70.72%). On the other side, C*. glabrata* ATCC2001, needed the higher dose, confirming a biofilm strong structure [99]. These findings have, also, been reported by other authors [94,100].

It is important to refer, that the obtained results regarding the biomass reductions do not match the susceptibilities' determinations (planktonics and biofilms), since high biomasses reductions were obtained, with lower (than MBEC) AmB concentrations for both formulations. It is known that, in situations of drug stress, some species/strains block the production and exportation of certain biofilm matrices' polymers (e.g. proteins in *C. glabrata*) [101] and that, in these cases,

subpopulations cells with increased tolerance to AmB or even persisters cells can arise, exclusively in the biofilms [65,67,102].

Although these facts are acknowledged, it is still extensively admitted that the biofilm drug resistance in *Candida* spp. remains to be totally unexplained and is most likely multifactorial in nature.

Table IV.2.3. Percentage of biofilm reduction closer to 50 when using AmB-Deox and AmB-L for reference species of *C. albicans, C. glabrata, C. parapsilosis and C. tropicalis*

Species	**[Drug] mg/L for biofilm reduction closer to 50%**			
	[AmB-Deox]	**% max biofilm reduction**	**[AmB-L]**	**% max biofilm reduction**
Candida albicans SC5314	0.25	68.56	0.5	70.72
Candida glabrata ATCC2001	1.5	51.56	1.5	48.86
Candida parapsilosis ATCC22019	1.5	34.64	1	43.78
Candida tropicalis ATCC750	0.25	89.58	1	50.66

Concluding, compared with conventional amphotericin B, the liposomal formulation offers a better safety profile in both adults and children and accumulates in tissue, which is therapeutically advantageous. Our results now show that AmB-L is a good option for the treatment of infections directly associated to *Candida* spp. biofilms cells. Continuous clinical observations are essential to measure the activity of AmB-L against yeasts, in order to detect strains with low drug susceptibility and thus supporting the most adequate choice of prompt antifungal treatment towards an improved prognosis to the patient.

References

1. Rodrigues, C.F.; Rodrigues, M.E.; Silva, S.; Henriques, M. Candida glabrata Biofilms: How Far Have We Come? *J. Fungi* **2017**, *3*, 11, doi:10.3390/JOF3010011.
2. Silva, S.; Rodrigues, C.; Araújo, D.; Rodrigues, M.; Henriques, M. Candida Species Biofilms' Antifungal Resistance. *J. Fungi* **2017**, *3*, 8, doi:10.3390/jof3010008.
3. Lass-Flörl, C. The changing face of epidemiology of invasive fungal disease in Europe. *Mycoses* **2009**, *52*, 197–205.
4. Tomičić, Z.; Zupan, J.; Matos, T.; Raspor, P.; Z, T.; J, Z.; T, M.; P, R. Probiotic yeast Saccharomyces boulardii (nom. nud.) modulates adhesive properties of Candida glabrata. *Med. Mycol.* **2016**, myw026, doi:10.1093/mmy/myw026.
5. Shields, R. K.; Nguyen, M. H.; Press, E. G.; Kwa, A. L.; Cheng, S.; Du, C.; Clancy, C. J. The presence of an FKS mutation rather than MIC is an independent risk factor for failure of echinocandin therapy among patients with invasive candidiasis due to Candida glabrata. *Antimicrob. Agents Chemother.* **2012**, *56*, 4862–9, doi:10.1128/AAC.00027-12.
6. Douglas, L. J. Candida biofilms and their role in infection. *Trends Microbiol.* **2003**, *11*, 30–6.
7. Ramage, G.; Rajendran, R.; Sherry, L.; Williams, C. Fungal biofilm resistance. *Int. J. Microbiol.* **2012**, *2012*, 528521, doi:10.1155/2012/528521.
8. Zhang, L.; She, X.; Merenstein, D.; Wang, C.; Hamilton, P.; Blackmon, A.; Hu, H.; Calderone, R.; Li, D. Fluconazole Resistance Patterns in Candida Species that Colonize Women with HIV Infection. *Curr. Ther. Res. Clin. Exp.* **2014**, *76*, 84–89, doi:10.1016/j.curtheres.2014.07.002.
9. Pierce, C. G.; Srinivasan, A.; Uppuluri, P.; Ramasubramanian, A. K.; López-Ribot, J. L. Antifungal therapy with an emphasis on biofilms. *Curr. Opin. Pharmacol.* **2013**, *13*, doi:10.1016/j.coph.2013.08.008.

10. Delattin, N.; Cammue, B. P. a; Thevissen, K. Reactive oxygen species-inducing antifungal agents and their activity against fungal biofilms. *Future Med. Chem.* **2014**, doi:10.4155/fmc.13.189.
11. Lemke, A.; Kiderlen, A. F.; Kayser, O. Amphotericin B. *Appl. Microbiol. Biotechnol.* **2005**, *68*, 151–162, doi:10.1007/s00253-005-1955-9.
12. Baginski, M.; Czub, J. Amphotericin B and its new derivatives-mode of action. *Curr Drug Metab* **2009**, *10*, 459–69.
13. Botero, M. C.; Puentes-Herrera, M.; Cortés, J. A. Lipid formulations of amphotericin. *Rev. Chilena Infectol.* **2014**, *31*, 518–27, doi:10.4067/S0716-10182014000500002.
14. Stephens, N.; Rawlings, B.; Caffrey, P. Streptomyces nodosus host strains optimized for polyene glycosylation engineering. *Biosci Biotechnol Biochem* **2012**, *76*, 384–387, doi:10.1271/bbb.110673.
15. Storm, G.; van Etten, E. Biopharmaceutical aspects of lipid formulations of amphotericin B. *Eur J Clin Microbiol Infect Dis* **1997**, *16*, 64–73.
16. Laniado-Laborin, R.; Cabrales-Vargas, M. Amphotericin B: side effects and toxicity. *Rev Iberoam Micol* **2009**, *26*, 223–7.
17. Azanza Perea, R.; Barberan, J. Anfotericina B forma liposomal: un perfil farmacocinético exclusivo. Una historia incabada. *Rev Esp Quim.* **2012**, *25*, 17–24.
18. Kshirsagar, N.; Pandya, S.; Kirodian, G.; Sanath, S. Liposomal drug delivery system from laboratory to clinic. *J Postgr. Med* **2005**, *51*, Suppl 1: S5-15.
19. Adler-Moore, J.; Proffitt, R. Amphotericin B lipid preparations: what are the differences? *Clin Microbiol Infect* **2008**, *14*, Suppl 4: 25-36.
20. Hamill, R. Amphotericin B formulations: a comparative review of efficacy and toxicity. *Drugs* **2013**, *73*, 919–34.
21. Patel, R. Amphotericin B colloidal dispersion. *Expert Opin Pharmacother* **2000**, *1*, 475–88.
22. Antoniadou, A.; Dupont, B. Lipid formulations of amphotericin B: where are we today? *J Mycol Med* **2005**, *14*, 230–8.
23. Berenguer, J.; Rodriguez-Tudela, J.; Richard, C.; Alvarez, M.; Sanz, M.; Gaztelurrutia, L.; Ayats, J.; Martinez-Suarez, J. Deep infections caused by Scedosporium prolificans. A report on 16 cases in Spain and a review of the literature. Scedosporium prolificans Spanish Study Group. *Med.* **1997**, *76*, 256–65.
24. Boutati, E.; Anaissie, E. Fusarium, a significant emerging pathogen in patients with hematologic malignancy: ten years' experience at a cancer center and implications for management. *Blood* **1997**, *90*, 999–1008.
25. Tritz, D.; Woods, G. Fatal disseminated infection with Aspergillus terreus in immunocompromised hosts. *Clin Infect Dis* **1993**, *16*, 118–22.
26. Montagna, M. T.; Lovero, G.; Coretti, C.; De Giglio, O.; Martinelli, D.; Bedini, A.; Delia, M.; Rosato, A.; Codeluppi, M.; Caggiano, G. In vitro activities of amphotericin B deoxycholate and liposomal amphotericin B against 604 clinical yeast isolates. *J. Med. Microbiol.* **2014**, 1–13, doi:10.1099/jmm.0.075507-0.
27. Moen, M. D.; Lyseng-Williamson, K. A.; Scott, L. J. Liposomal amphotericin B: a review of its use as empirical therapy in febrile neutropenia and in the treatment of invasive fungal infections. *Drugs* **2009**, *69*, 361–392, doi:10.2165/00003495-200969030-00010.
28. Faria-Ramos, I.; Neves-Maia, J.; Ricardo, E.; Santos-Antunes, J.; Silva, a T.; Costa-de-Oliveira, S.; Cantón, E.; Rodrigues, G.; Pina-Vaz, C. Species distribution and in vitro antifungal susceptibility profiles of yeast isolates from invasive infections during a Portuguese multicenter survey. *Eur. J. Clin. Microbiol. Infect. Dis.* **2014**, *33*, 2241–7, doi:10.1007/s10096-014-2194-8.
29. Krogh-Madsen, M.; Arendrup, M. C. M. C.; Heslet, L.; Knudsen, J. D. J. D. Amphotericin B and caspofungin resistance in Candida glabrata isolates recovered from a critically ill patient. *Clin. Infect. Dis.* **2006**, *42*, 938–944., doi:10.1086/500939.
30. Prażyńska, M.; Gospodarek, E. In vitro effect of amphotericin B on Candida albicans, Candida glabrata and Candida parapsilosis biofilm formation. *Mycopathologia* **2014**, *177*, 19–27, doi:10.1007/s11046-014-9727-7.
31. Kiraz, N.; Oz, Y. Species distribution and *in vitro* antifungal susceptibility of clinical *Candida* isolates from a university hospital in Turkey over a 5-year period. *Med. Mycol.* **2011**, *49*, 126–131, doi:10.3109/13693786.2010.503195.
32. Kawai, A.; Yamagishi, Y.; Mikamo, H. In vitro efficacy of liposomal amphotericin B, micafungin and fluconazole against non-albicans Candida species biofilms. *J. Infect. Chemother.* **2015**, *21*, 1–7, doi:10.1016/j.jiac.2015.05.007.
33. da Silva, B. V.; Silva, L. B.; de Oliveira, D. B. C.; da Silva, P. R.; Ferreira-Paim, K.; Andrade-Silva, L. E.; Silva-Vergara, M. L.; Andrade, A. A. Species Distribution, Virulence Factors, and Antifungal Susceptibility Among Candida parapsilosis Complex Isolates Recovered from Clinical Specimens. *Mycopathologia* **2015**, *180*, 333–343, doi:10.1007/s11046-015-9916-z.
34. EUCAST *Breakpoint tables for interpretation of MICs, Version 8.1, valid from 2017-03-01*;
35. Arendrup, M. C.; Arikan, S.; Barchiesi, F.; Bille, J.; Dannaoui, E.; Denning, D. W.; Donnelly, J. P.; Fegeler, W.;

Moore, C.; Richardson, M.; Schmalreck, A.; Velegraki, A.; Verweij, P. EUCAST Technical Note on the method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia – forming moulds. *ESCMID Tech. Notes* **2008**, *14*, 982–984, doi:10.1111/j.1469-0691.2008.02086.x.
36. Alastruey-Izquierdo, A.; Cuenca-Estrella, M. EUCAST and CLSI: How to assess in vitro susceptibility and clinical resistance. *Curr. Fungal Infect. Rep.* **2012**, *6*, 229–234, doi:10.1007/s12281-012-0100-3.
37. Rodrigues, C. F.; Gonçalves, B.; Rodrigues, M. E.; Silva, S.; Azeredo, J.; Henriques, M. The Effectiveness of Voriconazole in Therapy of Candida glabrata's Biofilms Oral Infections and Its Influence on the Matrix Composition and Gene Expression. *Mycopathologia* **2017**, *182*, 653–664, doi:10.1007/s11046-017-0135-7.
38. Rodrigues, C.F.; Henriques, M. Oral mucositis caused by Candida glabrata biofilms: failure of the concomitant use of fluconazole and ascorbic acid. *Ther. Adv. Infect. Dis.* **2017**, *1*, doi:10.1177/.
39. Pfaller, M. A.; Diekema, D. J. Epidemiology of invasive candidiasis: A persistent public health problem. *Clin. Microbiol. Rev.* **2007**, *20*, 133–163, doi:10.1128/CMR.00029-06.
40.Kanani, A.; Zaini, F.; kordbacheh, P.; Falahati, M.; Rezaie, S.; Daie, R.; Farahyar, S.; Safara, M.; Fateh, R.; Faghihloo, E.; Fattahi, A.; Heidari, M. Identification of Azole Resistance Markers in Clinical Isolates of Candida tropicalis Using cDNA-AFLP Method. *J. Clin. Lab. Anal.* **2015**, *272*, 266–272, doi:10.1002/jcla.21847.
41. Nabili, M.; Abdollahi Gohar, A.; Badali, H.; Mohammadi, R.; Moazeni, M. Amino acid substitutions in Erg11p of azole-resistant Candida glabrata: Possible effective substitutions and homology modelling. *J. Glob. Antimicrob. Resist.* **2016**, *5*, 42–46, doi:10.1016/j.jgar.2016.03.003.
42. Pappas, P. G.; Kauffman, C. A.; Andes, D. R.; Clancy, C. J.; Marr, K. A.; Ostrosky-Zeichner, L.; Reboli, A. C.; Schuster, M. G.; Vazquez, J. A.; Walsh, T. J.; Zaoutis, T. E.; Sobel, J. D. Clinical Practice Guideline for the Management of Candidiasis: 2016 Update by the Infectious Diseases Society of America. *Clin. Infect. Dis.* **2015**, *62*, e1–e50, doi:10.1093/cid/civ933.
43. McCarty, T. P.; Pappas, P. G. Invasive Candidiasis. *Infect. Dis. Clin. North Am.* 2016, *30*.
44. Pfaller, M. A.; Messer, S. A.; Rhomberg, P. R.; Jones, R. N.; Castanheira, M. Activity of a long-acting echinocandin, CD101, determined using CLSI and EUCAST reference methods, against Candida and Aspergillus spp., including echinocandin- and azole-resistant isolates. *J. Antimicrob. Chemother.* **2016**, *101*, dkw214, doi:10.1093/jac/dkw214.
45. Sanguinetti, M.; Posteraro, B.; Lass-Flörl, C. Antifungal drug resistance among Candida species: Mechanisms and clinical impact. *Mycoses* **2015**, *58*, doi:10.1111/myc.12330.
46. Mesa-Arango, a. C.; Trevijano-Contador, N.; Roman, E.; Sanchez-Fresneda, R.; Casas, C.; Herrero, E.; Arguelles, J. C.; Pla, J.; Cuenca-Estrella, M.; Zaragoza, O. The Production of Reactive Oxygen Species Is a Universal Action Mechanism of Amphotericin B against Pathogenic Yeasts and Contributes to the Fungicidal Effect of This Drug. *Antimicrob. Agents Chemother.* **2014**, *58*, 6627–6638, doi:10.1128/AAC.03570-14.
47. Neofytos, D.; Lombardi, L. R.; Shields, R. K.; Ostrander, D.; Warren, L.; Nguyen, M. H.; Thompson, C. B.; Marr, K. A. Administration of voriconazole in patients with renal dysfunction. *Clin. Infect. Dis.* **2012**, *54*, 913–921, doi:10.1093/cid/cir969.
48. Linder, N.; Klinger, G.; Shalit, I.; Levy, I.; Ashkenazi, S.; Haski, G.; Levit, O.; Sirota, L. Treatment of candidaemia in premature infants: comparison of three amphotericin B preparations. *J. Antimicrob. Chemother.* **2003**, *52*, 663–7, doi:10.1093/jac/dkg419.
49. Ostrosky-Zeichner, L.; Marr, K. A.; Rex, J. H.; Cohen, S. H. Amphotericin B: Time for a new "gold standard." *Clin. Infect. Dis.* **2003**, *37*, 415–425, doi:10.1086/376634.
50. Safdar, A.; Ma, J.; Saliba, F.; Dupont, B.; Wingard, J.; Hachem, R.; Mattiuzzi, G.; Chandrasekar, P.; Kontoyiannis, D.; Rolston, K.; Walsh, T.; Champlin, R.; Raad, I. Drug-induced nephrotoxicity caused by amphotericin B lipid complex and liposomal amphotericin B: a review and meta-analysis. *Med.* **2010**, *89*, 236–44.
51. Coenye, T.; Bjarnsholt, T. The complexity of microbial biofilm research – an introduction to the 3 rd Thematic Issue on Biofilms. *Pathog. Dis.* **2016**.
52. Fielding, R.; Smith, P.; Wang, L.; Porter, J.; Guo, L. Comparative pharmacokinetics of amphotericin B after administration of a novel colloidal delivery system, ABCD, and a conventional formulation to rats. *Antimicrob Agents Chemother* **1991**, *35*, 1208–13.
53. Carrillo-Muñoz, A.; Quinds, G.; Tur, C.; Ruesga, M.; Miranda, Y.; del Valle, O.; Cossum, P.; Wallace, T. In-vitro antifungal activity of liposomal nystatin in comparison with nystatin, amphotericin B cholesteryl sulphate, liposomal amphotericin B, amphotericin B lipid complex, amphotericin B desoxycholate, fluconazole and itraconazole. *J Antimicrob Chemother.* **1999**, *44*, 397–401.
54. Gonzalez, G. M.; Elizondo, M.; Ayala, J. Trends in species distribution and susceptibility of bloodstream isolates of Candida collected in Monterrey, Mexico, to seven antifungal agents: Results of a 3-year (2004 to 2007) surveillance study. *J. Clin. Microbiol.* **2008**, *46*, 2902–2905, doi:10.1128/JCM.00937-08.
55. Hull, C. M.; Bader, O.; Parker, J. E.; Weig, M.; Gross, U.; Warrilow, A. G. S.; Kelly, D. E.; Kelly, S. L. Two clinical isolates of Candida glabrata exhibiting reduced sensitivity to amphotericin B both harbor mutations in ERG2. *Antimicrob. Agents Chemother.* **2012**, *56*, 6417–6421, doi:10.1128/AAC.01145-12.

56. Hull, C. M.; Parker, J. E.; Bader, O.; Weig, M.; Gross, U.; Warrilow, A. G. S.; Kelly, D. E.; Kelly, S. L. Facultative sterol uptake in an ergosterol-deficient clinical isolate of candida glabrata harboring a missense mutation in ERG11 and exhibiting cross-resistance to azoles and amphotericin B. *Antimicrob. Agents Chemother.* **2012**, *56*, 4223–4232, doi:10.1128/AAC.06253-11.
57. Pfaller, M. A.; Messer, S. A.; Hollis, R. J.; Jones, R. N.; Diekema, D. J. In Vitro Activities of Ravuconazole and Voriconazole Compared with Those of Four Approved Systemic Antifungal Agents against 6 , 970 Clinical Isolates of Candida spp . *Antimicrob Agents Chemother* **2002**, *46*, 1723–1727, doi:10.1128/AAC.46.6.1723.
58. Groll, A. H.; Giri, N.; Petraitis, V.; Petraitiene, R.; Candelario, M.; Bacher, J. S.; Piscitelli, S. C.; Walsh, T. J. Comparative Efficacy and Distribution of Lipid Formulations of Amphotericin B in Experimental *Candida albicans* Infection of the Central Nervous System. *J. Infect. Dis.* **2000**, *182*, 274–282, doi:10.1086/315643.
59. Anaissie, E.; Paetznick, V. L.; Proffitt, R.; Adler-Moore, J.; Bodey, G. Comparison of the in vitro antifungal activity of free and liposome-encapsulated amphotericin B. *Eur J Clin Microbiol Infect Dis.* **1991**, *10*, 665–8.
60. Jessup, C.; Reyes, G.; Fothergill, A.; McCarthy, D.; M, R.; Messer, M.; Pfaller, M.; Ghannoum, M. A head-on comparison of the in vitro antifungal activity of conventional and lipid-based amphotericin B: a multicenter study. *J Chemother.* **2000**, *12*, 22–9.
61. Lass-Flörl, C.; Mayr, A.; Perkhofer, S.; Hinterberger, G.; Hausdorfer, J.; Speth, C.; Fille, M. Activities of antifungal agents against yeasts and filamentous fungi: Assessment according to the methodology of the European Committee on Antimicrobial Susceptibility Testing. *Antimicrob. Agents Chemother.* **2008**, *52*, 3637–3641, doi:10.1128/AAC.00662-08.
62. Ito, J. I.; Hooshmand-Rad, R. Treatment of Candida infections with amphotericin B lipid complex. *Clin. Infect. Dis.* **2005**, *40 Suppl 6*, S384-91, doi:10.1086/429330.
63. Sun, J.; Li, Z.; Chu, H.; Guo, J.; Jiang, G.; Qi, Q. Candida albicans Amphotericin B-Tolerant Persister Formation is Closely Related to Surface Adhesion. *Mycopathologia* **2016**, *181*, 41–49, doi:10.1007/s11046-015-9894-1.
64. Boucherit, Z.; Seksek, O.; Bolard, J. Dormancy of Candida albicans cells in the presence of the polyene antibiotic amphotericin B: simple demonstration by flow cytometry. *Med. Mycol.* **2007**, *45*, 525–33, doi:10.1080/13693780701487821.
65. LaFleur, M. D.; Kumamoto, C. A.; Lewis, K. Candida albicans biofilms produce antifungal-tolerant persister cells. *Antimicrob. Agents Chemother.* **2006**, *50*, 3839–3846, doi:10.1128/AAC.00684-06.
66. LaFleur, M.; Qingguo, Q.; Lewis, K. Patients with long-term oral carriage harbor high-persister mutants of Candida albicans. *Antimicrob. Agents Chemother.* **2010**, *54*, 39–44, doi:10.1128/AAC.00860-09.
67. Al-Dhaheri, R. S. R. S.; Douglas, L. J. J. Absence of amphotericin B-tolerant persister cells in biofilms of some Candida species. *Antimicrob. Agents Chemother.* **2008**, *52*, 1884–1887, doi:10.1128/AAC.01473-07.
68. Al-Dhaheri, R. S.; Douglas, L. J. Apoptosis in Candida biofilms exposed to amphotericin B. *J. Med. Microbiol.* **2010**, *59*, 149–157, doi:10.1099/jmm.0.015784-0.
69. Bink, A.; Vandenbosch, D.; Coenye, T.; Nelis, H.; Cammue, B. P. A.; Thevissen, K. Superoxide dismutases are involved in Candida albicans biofilm persistence against miconazole. *Antimicrob. Agents Chemother.* **2011**, *55*, 4033–4037, doi:10.1128/AAC.00280-11.
70. Dawson, C. C.; Intapa, C.; Jabra-Rizk, M. A. "Persisters": Survival at the cellular level. *PLoS Pathog.* **2011**, *7*, 2–4, doi:10.1371/journal.ppat.1002121.
71. Uppuluri, P.; Chaturvedi, A.; Srinivasan, G.; Banerjee, M.; Ramasubramaniam, A.; et al Dispersion as an important step in the Candida albicans biofilm developmental cycle. *PLoS Pathog* **2010**, 6:e1000828.
72. Lal, P.; Sharma, D.; Pruthi, P.; Pruthi, V. Exopolysaccharide analysis of biofilm-forming Candida albicans. *J. Appl. Microbiol.* **2010**, *109*, 128–136, doi:10.1111/j.1365-2672.2009.04634.x.
73. Fonseca, E.; Silva, S.; Rodrigues, C.F.; Alves, C.; Azeredo, J.; Henriques, M. Effects of fluconazole on Candida glabrata biofilms and its relationship with ABC transporter gene expression. *Biofouling* **2014**, *30*, 447–57, doi:10.1080/08927014.2014.886108.
74. Hawser, S. P.; Douglas, L. J. Resistance of Candida albicans biofilms to antifungal agents in vitro. *Antimicrob. Agents Chemother.* **1995**, *39*, 2128–2131.
75. Taff, H.; Nett, J.; Andes, D. Comparative analysis of Candida biofilm quantitation assays. *Med Mycol* **2012**, *50*, 214–218.
76. Heffner, D.; Franklin, W. Endocarditis caused by Torulopsis glabrata. *Am J Clin Pathol* **1978**, *70*, 420–423.
77. Tumbarello, M.; Fiori, B.; Trecarichi, E. M.; Posteraro, P.; Losito, A. R.; de Luca, A.; Sanguinetti, M.; Fadda, G.; Cauda, R.; Posteraro, B.; M, T.; B, F.; EM, T.; P, P.; Al, L.; et al; Tumbarello, M.; Fiori, B.; Trecarichi, E. M.; Posteraro, P.; Losito, A. R.; de Luca, A.; Sanguinetti, M.; Fadda, G.; Cauda, R.; Posteraro, B. Risk factors and outcomes of candidemia caused by biofilm-forming isolates in a tertiary care hospital. *PLoS One* **2012**, *7*, 1–9, doi:10.1371/journal.pone.0033705.
78. Vila, T. V. M.; Rozental, S. Biofilm formation as a pathogenicity factor of medically important fungi. In *Fungal*

Pathogenicity, 2016; pp. 1–24.
79. López-Ribot, J. L. Candida albicans biofilms: more than filamentation. *Curr. Biol.* **2005**, *15*, R453-5, doi:10.1016/j.cub.2005.06.020.
80. Mitchell, K. F.; Taff, H. T.; Cuevas, M. A.; Reinicke, E. L.; Sanchez, H.; Andes, D. R. Role of matrix β-1,3-glucan in antifungal resistance of non-albicans Candida biofilms. *Antimicrob. Agents Chemother.* **2013**, *57*, 1918–1920.
81. Rodrigues, C. F.; Silva, S.; Azeredo, J.; Henriques, M.; Henrique, M.; Henriques, M. Detection and Quantification of Fluconazole Within Candida glabrata Biofilms. *Mycopathologia* **2015**, *179*, 391–5, doi:10.1007/s11046-015-9862-9.
82. Wingard, J.; White, M.; Anaissie, E.; Raffalli, J.; Goodman, J.; Arrieta, A.; L Amph/ABLC Collaborative Study Group; et al A randomized, double-blind comparative trial evaluating the safety of liposomal amphotericin B versus amphotericin B lipid complex in the empirical treatment of febrile neutropenia. *Clin Infect Dis.* **2000**, *64*, 1155–1163., doi:10.1086/317451.
83. Astellas Pharma US, I. *AmBisome (amphotericin B) liposome for injection Northbrook, IL*;
84. Vandeputte, P.; Tronchin, G.; Larcher, G.; Ernoult, E.; Bergès, T.; Chabasse, D.; Bouchara, J.-P. A nonsense mutation in the ERG6 gene leads to reduced susceptibility to polyenes in a clinical isolate of Candida glabrata. *Antimicrob. Agents Chemother.* **2008**, *52*, 3701–9, doi:10.1128/AAC.00423-08.
85. Vandeputte, P.; Tronchin, G.; Bergès, T.; Hennequin, C.; Chabasse, D.; Bouchara, J.-P. Reduced susceptibility to polyenes associated with a missense mutation in the ERG6 gene in a clinical isolate of Candida glabrata with pseudohyphal growth. *Antimicrob. Agents Chemother.* **2007**, *51*, 982–90, doi:10.1128/AAC.01510-06.
86. Ferrari, S.; Sanguinetti, M.; De Bernardis, F.; Torelli, R.; Posteraro, B.; Vandeputte, P.; Sanglard, D. Loss of mitochondrial functions associated with azole resistance in Candida glabrata results in enhanced virulence in mice. *Antimicrob. Agents Chemother.* **2011**, *55*, 1852–60, doi:10.1128/AAC.01271-10.
87. Choi, H. W.; Shin, J. H.; Jung, S. I.; Park, K. H.; Cho, D.; Kee, S. J.; Shin, M. G.; Suh, S. P.; Ryang, D. W. Species-specific differences in the susceptibilities of biofilms formed by Candida bloodstream isolates to echinocandin antifungals. *Antimicrob. Agents Chemother.* **2007**, *51*, 1520–1523, doi:10.1128/AAC.01141-06.
88. Kuhn, D. M.; George, T.; Chandra, J.; Mukherjee, P. K.; Ghannoum, M. A. Antifungal Susceptibility of Candida Biofilms: Unique Efficacy of Amphotericin B Lipid Formulations and Echinocandins. *Antimicrob Agents Chemother* **2002**, *46*, 1773–1780, doi:10.1128/AAC.46.6.1773.
89. Liao, Z. Bin; ZhangGuan, X. Z.; Zhu, Z. Y.; Yao, X. W.; Yang, Y.; Jiang, Y. Y.; Cao, Y. Y. Enhancement of the antibiofilm activity of amphotericin B by polyamine biosynthesis inhibitors. *Int. J. Antimicrob. Agents* **2014**, *46*, 45–52, doi:10.1016/j.ijantimicag.2015.02.021.
90. Luiz, R. L. F.; Vila, T. V. M.; de Mello, J. C. P.; Nakamura, C. V.; Rozental, S.; Ishida, K. Proanthocyanidins polymeric tannin from Stryphnodendron adstringens are active against Candida albicans biofilms. *BMC Complement. Altern. Med.* **2015**, *15*, 68, doi:10.1186/s12906-015-0597-4.
91. Kvasnickova, E.; Matatkova, O.; Cejkova, A.; Masak, J. Evaluation of baicalein, chitosan and usnic acid effect on Candida parapsilosis and Candida krusei biofilm using a Cellavista device. *J. Microbiol. Methods* **2015**, *118*, 106–112, doi:10.1016/j.mimet.2015.09.002.
92. Mahl, C. D.; Behling, C. S.; Hackenhaar, F. S.; de Carvalho E Silva, M. N.; Putti, J.; Salomon, T. B.; Alves, S. H.; Fuentefria, A.; Benfato, M. S. Induction of ROS generation by fluconazole in Candida glabrata: activation of antioxidant enzymes and oxidative DNA damage. *Diagn. Microbiol. Infect. Dis.* **2015**, *82*, 203–8, doi:10.1016/j.diagmicrobio.2015.03.019.
93. Marcos-Zambrano, L.; Escribano, P.; Bouza, E.; Guinea, J. Comparison of the antifungal activity of micafungin and amphotericin B against Candida tropicalis biofilms. *J Antimicrob Chemother.* **2016**.
94. Zahran, K. M.; Agban, M. N.; Ahmed, S. H.; Hassan, E. A.; Sabet, M. A. Patterns of candida biofilm on intrauterine devices. *J. Med. Microbiol.* **2015**, *64*, 375–381, doi:10.1099/jmm.0.000042.
95. Shanmughapriya, S.; Sornakumari, H.; Lency, A.; Kavitha, S.; Natarajaseenivasan, K. Synergistic effect of amphotericin B and tyrosol on biofilm formed by Candida krusei and Candida tropicalis from intrauterine device users. *Med. Mycol.* **2014**, *52*, 853–861, doi:10.1093/mmy/myu046.
96. Maiolo, E. M.; Tafin, U. F.; Borens, O.; Trampuz, A. Activities of fluconazole, caspofungin, anidulafungin, and amphotericin b on planktonic and biofilm candida species determined by microcalorimetry. *Antimicrob. Agents Chemother.* **2014**, *58*, 2709–2717, doi:10.1128/AAC.00057-14.
97. Aslan, H.; Gulmez, D. Investigation of the correlation between biofilm forming ability of urinary Candida isolates with the use of urinary catheters and change of antifungal susceptibility in the presence of biofilm. *Mikrobiyol Bul.* **2016**, *50*, 256–65.
98. Seneviratne, C. J.; Wang, Y.; Jin, L.; Abiko, Y.; Samaranayake, L. P.; Jayampath Seneviratne, C.; Wang, Y.; Jin, L.; Abiko, Y.; Samaranayake, L. P. Proteomics of drug resistance in candida glabrata biofilms. *Proteomics* **2010**, *10*, 1444–1454, doi:10.1002/pmic.200900611.
99. Rodrigues, C. F.; Silva, S.; Azeredo, J.; Henriques, M. Candida glabrata's recurrent infections: biofilm formation

during Amphotericin B treatment. *Lett. Appl. Microbiol.* **2016**, *63*, 77–81, doi:10.1111/lam.12600.
100. Basso, L. R.; Gast, C. E.; Mao, Y.; Wong, B. Fluconazole Transport into Candida albicans Secretory Vesicles by the Membrane Proteins Cdr1p, Cdr2p, and Mdr1p. *Eukaryot Cell* **2010**, *9*, 960–970, doi:10.1016/j.jsps.2013.12.007.
101. Miyazaki, T.; Kohno, S. ER stress response mechanisms in the pathogenic yeast Candida glabrata and their roles in virulence. *Virulence* **2014**, *5*, 365–70, doi:10.4161/viru.27373.
102. Prasanna, K. D.; Suci, P. A.; Miller, R. L.; Nelson, R. D.; Tyler, B. J. A Small Subpopulation of Blastospores in Candida albicans Biofilms Exhibit Resistance to Amphotericin B Associated with Differential Regulation of Ergosterol and B-1,6-Glucan Pathway Genes. *Antimicrob. Agents Chemother.* **2006**, *50*, 3708–3716, doi:10.1128/AAC.00997-06.

IV.3

Association of Posaconazole and Amphotericin B in the treatment of biofilms of *Candida glabrata*

ABSTRACT

Purpose: The incidence of candidaemia is increasing in the last years, especially among immunosuppressed individuals. The disease is severe and difficult to treat due to the general debilitated state of the patients and the *Candida* spp. resistance to drug treatments. Among all species, *Candida glabrata* is one of the most resistant, especially to the azole class. It is thus, important to search for alternative therapies, as the combination of antifungals from different classes.

Methodology: In this work, the minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC) and minimum biofilm eradicatory concentration (MBEC) were determined for posaconazole (Pcz) and amphotericin B (AmB). The drug combinations of both drugs were evaluated on 24-h-formed biofilms of *C. glabrata* ATCC2001, through XTT assay, colony forming units (CFU), crystal violet. Moreover, the fractional inhibitory concentration index (FICI) was determined.

Results: C. glabrata demonstrated to have a better susceptibility to AmB than to Pcz. Also, AmB confirmed to have a higher capacity to reduce biofilm cells, than Pcz, but both drugs revealed a good capacity in eliminating the biomass. In the majority of the tested combinations, the interactions were determined as indifferent (FICI ≤ 4).

Conclusion: The association of the drugs, does not seem to bring a clear advantage in the treatment of 24-h-biofilms of *C. glabrata* ATCC2001.

Keywords: *Candida*, posaconazole, amphotericin B, biofilms, matrix, antifungal drug, antifungal association, resistance, gene expression, *Candida glabrata*.

IV.1.3 Introduction

Systemic fungal infections caused by *Candida* spp. have raised in the last decades, in parallel with the number of immunocompromised patients [1–3]. *Candida glabrata* is currently the second most common cause of candidaemia in the United States and the third in Europe [4,5]. Although not having the capacity to form filaments or to secret proteases, *C. glabrata* has other virulence factor*s*, as secretion of phospholipases, lipases, and haemolysins, and specially their ability to form biofilms [2,6]. Biofilms are surface-associated communities of microorganisms embedded in an extracellular matrix which confers protection and consequently a major resistance to antifungal therapy [7,8]. The increase of *C. glabrata* infections is also related to an inherent low susceptibility to azoles [9] and can also be a result of rare mutations that are selected by drug pressure [10]. Accordingly, the cases of fluconazole-refractory disease (resistance to fluconazole) related to *C. glabrata* are common in candidiasis. So, the treatment of these infections rely on other azoles (e.g. voriconazole and posaconazole - Pcz) or other classes of antifungal agents, such as polyenes (e.g. amphotericin B - AmB) and echinocandins [3]. However, resistance to polyenes has been reported on *C. glabrata* [11–15], as well as, resistance or low susceptibility to Pcz [16–18].

Pcz is a lipophilic broad-spectrum triazole drug, introduced in 2007 [19,20] and the most recent guidelines for the treatment of fungal infections indicate Pcz as a valuable choice in therapy of *Candida* spp. intravascular infections, oropharyngeal or esophageal candidiasis [3,21]. AmB is a polyene produced by *Streptomyces nodosus* [22,23], characterized by a macrocyclic ring lactone, that has demonstrated to be effective for the treatment candidemia in nonneutropenic patients and urinary tract infections, specially due to *C. glabrata* and *C. krusei* [3]. Therefore, the main aim of this research was to evaluate the *in vitro* activity of the combination of one azole (Pcz) and one polyene (AmB) against *C. glabrata* mature biofilms.

IV.2.3 Material and Methods

IV.2.3.1 Organism and growth conditions

The reference strain from the American Type Culture Collection, *C. glabrata* ATCC2001, was used in this study. For each experiment, *C. glabrata* ATCC2001 was subcultured on Sabouraud dextrose agar (SDA) (Merck, Darmstadt, Germany), during 24 h at 37°C. Cells were then inoculated in Sabouraud dextrose broth (SDB) (Merck, Darmstadt, Germany) and incubated for 18 h at 37°C under agitation at 120 rpm. After incubation, the cells were harvested by centrifugation at 3000 *g* for 10 min at 4°C and washed twice with Phosphate Buffered Saline (PBS 0.1 *M*, pH=7.5). Pellets

were then suspended in RPMI-1640 (pH=7, Sigma-Aldrich, Roswell Park) and the cellular density was adjusted to $1x10^5$ cells/mL, using a Neubauer counting chamber.

IV.2.3.2Antifungal drugs

Posaconazole was kindly provided by Merck®, S.A. in its pure compound. Amphotericin B was purchased in Sigma® (Sigma-Aldrich, Roswell Park). Aliquots of 5000 mg/L were prepared using dimethyl-sulfoxide (DMSO), for both drugs, and the final concentrations were prepared in RPMI-1640.

IV.2.3.3Minimum Inhibitory Concentrations (MICs)

The tested concentrations of Pcz and AmB were prepared in RPMI-1640. The inoculum was prepared by suspending five distinct colonies, ≥1 mm diameter from 24 h cultures, in at least 3 mL of sterile distilled water. Then, the inoculum was suspended by vigorous shaking on a vortex mixer for 15 s and the cell density was adjusted to the density of a 0.5 McFarland standard and adding sterile distilled water as required, giving a yeast suspension of 1-5 x 10^6 colony forming units (CFUs)/mL. A working suspension was prepared by a dilution of the standardised suspension in sterile distilled water to yield 1-5 x 10^5 CFU/mL. The 96-well-plate was prepared with 100 µL of cell suspension and 100 µL of both antifungal agents (0.2 to 1 mg/L for Pcz and 0.25 to 2 for AmB – 2 x concentrated) and incubated at 37°C, during 18-48 h. Positive controls without Pcz and AmB were also performed. The results were visualized by spectrometry at 530 nm.

IV.2.3.4Minimum Fungicidal Concentration (MFC)

In addition to the previous step, 20 µL of each cell suspension treated with Pcz and AmB was recovered to a new well and serial decimal dilutions in PBS were plated onto SDA. Agar plates were incubated for 24 h at 37 °C, and the total number of CFUs was determined. The results were presented per Log_{10} CFU per area (Log_{10} CFUs/cm^2) [24].

IV.2.3.5Minimum Biofilm Eradication Concentration (MBEC)

Standardized cell suspensions (200 µL) were placed into selected wells of 96-wells polystyrene microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium). RPMI-1640 was used without cells, but with antifungal agent, as a negative control. As positive control cell suspensions were tested without antifungal agent. At 24 h, 100 µL of RPMI-1640 was removed and an equal volume of

fresh RPMI-1640 plus the antifungal concentration were added (Pcz: 200; 300 and 600 mg/L; AmB: 0.5; 1; 2; and 4 mg/L, 2x concentrated). The plates were incubated at 37°C for more 24 h at 120 rpm. The number of cultivable cells on biofilms was determined by the enumeration of CFUs. For that, after the period of biofilm formation, all medium was aspired and the biofilms washed once with 200 μL of PBS to remove non-adherent cells. Then, biofilms were scraped from the wells and the suspensions were vigorously vortexed for 2 min to disaggregate cells from the matrix. Serial decimal dilutions in PBS were plated on SDA and incubated for 24 h at 37°C. The results were presented as Log_{10} CFU per area (Log_{10} CFUs/cm^2) [24].

IV.2.3.6XTT reduction and checkerboard assay

Biofilms were formed as explained previously. The metabolic activity of the biofilms was measured by assaying 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction, a reaction catalyzed by mitochondrial dehydrogenases, as described before [25]. Briefly, biofilms were washed with sterile ultrapure water and were then incubated with 100 μg/mL XTT and 10 μg/mL in PMS, at 37°C for 3h at 120 rpm. After, the optical density (OD) was measured at 490 nm using a microtitre plate reader. The metabolic activity of each drug combination was compared with that of the drug-free biofilms and drug-alone biofilms (control biofilms).

IV.2.3.7Interpretation of drug combination interaction

Drug combination (200 mg/L of Pcz and 0.5 mg/L of AmB) interaction was classified on the basis of the fractional inhibitory concentration index (FICI) [26,27]. The FICI was calculated by the formula: $FICI=(A_c/A_a)+(B_c/B_a)$, where A_c and B_c are the metabolic activity of the biofilm cells with antifungal drugs in combination, and A_a and B_a are the metabolic activity of the biofilm cells with antifungal drugs A and B alone. The interaction was defined as synergistic if the FICI was ≤0.5, additive if FICI was >0.5 and ≤1, indifferent if the FICI was >1 and ≤4, and antagonistic if the FICI was >4.0.

IV.2.3.8Biofilm total biomass quantification – Crystal Violet Staining

Total biofilm biomass was quantified by Crystal Violet (CV) staining [24]. After biofilms formation, the medium was aspirated and non-adherent cells removed by washing the biofilms with sterile ultra-pure water. Then, biofilms were fixed with 200 μL methanol, which was removed after 15 min of contact. The microtiter plates were allowed to dry at room temperature, and 200 μL of CV (1%

v/v) were added to each well and incubated for 5 min. The wells were then gently washed twice with sterile, ultra-pure water and 200 µL of acetic acid (33% v/v) were added to release and dissolve the stain. The absorbance of the obtained solution was read in triplicate in a microtiter plate reader (Bio-Tek Synergy HT, Izasa, Lisbon, Portugal) at 570 nm. The results were presented as absorbance per unit area (Abs/cm^2).

IV.2.3.9 Statistical Analysis

Experiments were repeated three times in independent assays. Results were compared using one-way ANOVA, Dunnet's post hoc multiple comparisons test, using GraphPad™ Prism 7™ (CA, USA) software. All tests were performed with a confidence level of 95%.

IV.3.3. Results and Discussion

Systemic candidaemia is a growing problem worldwide, especially in critically immunocompromised patients [2,28]. It is associated to severe rates of morbidity, mortality and high economic costs [1,29], so the use of a proper therapeutics for this fungal infection is crucial to achieve a clinical cure. Accordingly, the two drugs studied in this work were chosen since both are used in the treatment of systemic candidaemia and have different modes of action [3].

Table IV.3.1 shows the results on the determinations of MIC, MFC and MBECs for Pcz and AmB for *C. glabrata* ATCC2001.

Table IV.3.1. MICs, MFCs and MBECs determined for posaconazole and amphotericin B for *C. glabrata* ATCC2001

Drug	MIC (mg/L)	MFC (mg/L)	MBEC (mg/L)
Posaconazole	0.7	0.8-0.9	> 300
Amphotericin B	0.25	1-2	2-4

Although the 2017 EUCAST guidelines do not specify epidemiological cut-off value (ECOFFs) for *C. glabrata* strains regarding Pcz, it is indicated that, for this species, these values are in general higher than for *C. albicans* (sensible ≤ 0.064 mg/L; resistant > 0.064 mg/L). Thus, the value observed (0.7 mg/L) seems to indicate a tolerant profile [30]. Regarding the AmB MICs, EUCAST points out that strains with values of AmB ≤1 mg/L should be considered sensible, which was the case, since MIC was 0.25 mg/L (Table IV.3.1) [30]. Surprisingly, the MIC and MFC determinations for Pcz showed that this drug has a good activity in planktonic cells. Comparing the MIC and the MFC value, they are approximately the same (0.8-0.9 mg/L vs 0.7 mg/L). In a recent work published by our group, the MFC for fluconazole (Flu) and voriconazole (Vcz) for *C. glabrata*

ATCC2001 was almost 200 times and 4 times higher than the MIC, respectively [24]. So, the result for Pcz shows an enhanced effectiveness to eliminate planktonic cells, in comparison to Flu and Vcz [24]. Furthermore, the similar MICs determined for Pcz and Vcz (0.7 and 0.5 mg/L, respectively) confirm the parallel Pcz *in vitro* activity against *Candida* spp. that have been reported for both drugs [31].

Figure IV.3.1 shows the effect of Pcz (A) and AmB (C) on *C. glabrata* ATCC2001 biofilms' cells and biomass. Regarding analysis of biofilm cells (enumeration of CFU), Pcz showed to reduce 1 Log_{10} CFU/cm^2 of the 24-h *C. glabrata* ATCC2001 biofilm cells, when using 200 mg/L ($P<0.0005$). Curiously, increasing the dose there was not an improvement in biofilm cells reduction (Figure IV.3.1(A)). On the other side, AmB displayed a better performance, reducing 2 Log_{10} CFU/cm^2 from 2 mg/L (Figure IV.3.1(C)). Concerning the biomass reduction, both drugs revealed to be good biofilm reducers. Pcz exhibited a statistically significant higher biomass drop with the lower concentration (77.6%, $P< 0.0001$), when in comparison with 2 mg/L of AmB (64.2%, $P<0.0001$) (Figure IV.3.1 B and D)

The azoles, as Pcz, target the ergosterol biosynthetic pathway, specifically the 14-α sterol demethylases that are encoded by the *ERG11* gene. Thus, these drugs are responsible for blocking the capacity to build and renew sterols in the cellular membranes, changing membrane fluidity and function of vital processes such as signaling, transport, exocytosis, and endocytosis [2,6]. The polyenes, as AmB, bind to the ergosterol of the fungal cell wall establishing transmembrane aggregates pores and causing membrane depolarization and membrane permeability, which leads to osmotic imbalance and finally cell death [32–34].

Although not considered a first-line drug for primary candidiasis therapy, Pcz is used in cases of *Candida* spp. infections as stepping-down therapy for isolates that are susceptible to those agents but not susceptible to Flu (fluconazole-refractory disease) [3]. The concomitant use of azoles and polyenes has been evaluated in some reports in other species [3,35–41]. With the purpose of evaluating the antifungal effect (synergism, additive, indifferent or antagonism) of the association of Pcz and AmB, clinical concentrations were applied in a matured biofilm of *C. glabrata* ATCC2001. It is important to note that the concentrations used were the MICs of Pcz and AmB and doses similar to the protocols of doses used in invasive candidiasis [3].

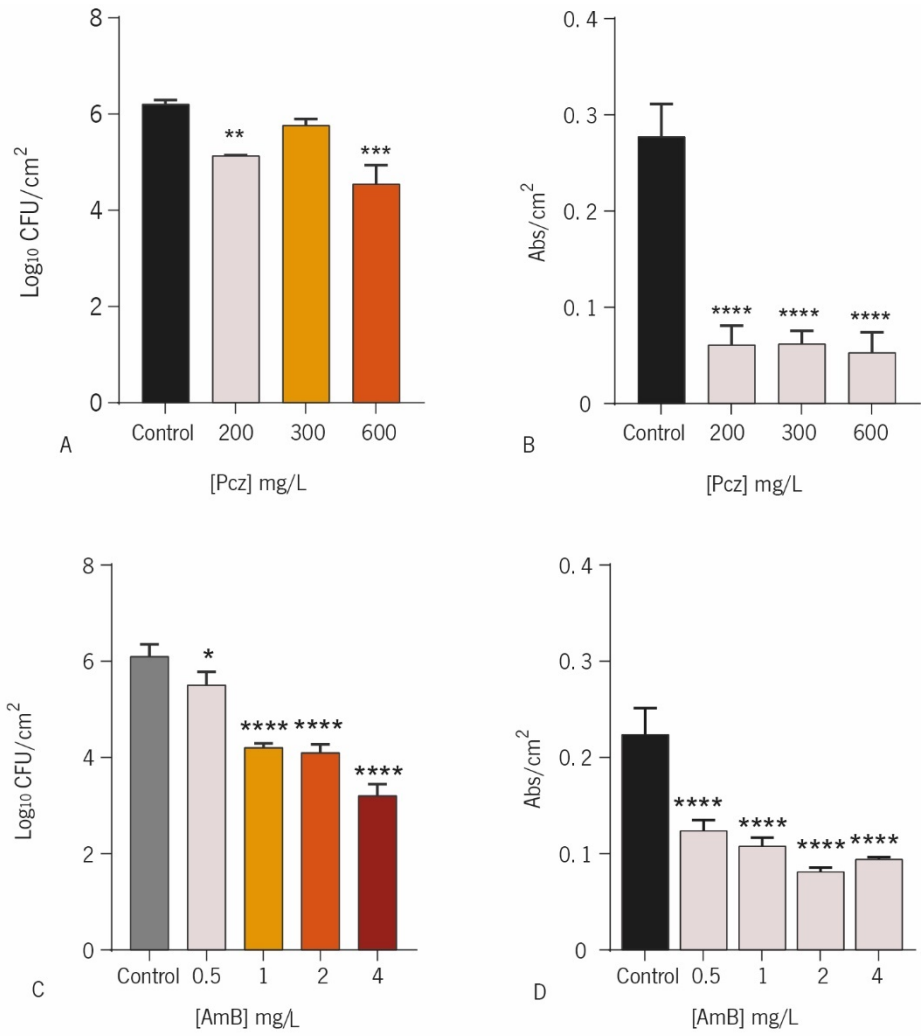


Figure IV.3.1. Effect of posaconazole (Pcz) and amphotericin B (AmB) on *C. glabrata* ATCC2001 biofilms. Mean values of the logarithm of colony forming units normalized by unit of area (Log_{10} CFU/cm^2) (A and C). Crystal Violet in a 48-hour-biofilm of *C. glabrata* ATCC2001, with and without posaconazole (200; 300; 600 mg/L) (B), amphotericin B (D) (0.25; 0.5; 1 mg/L). The quantification of the biomass is presented by Abs/cm^2 *(* P<0.05; ** P<0.001; *** P<0.0005; **** P< 0.0001).*

Figure IV.3.2A displays the checkerboard of the combinatory effect of different concentrations of Pcz and AmB. The values presented are determinations of the metabolic activity of the biofilms, determined through the XTT assay. These results were corroboratory of the CFU counts, since, generally, AmB alone showed to have higher capacity to decrease the metabolic activity of the biofilm cells, than Pcz alone (Figure IV.3.2A). Also, in terms of metabolic activity, only one combination was able to be 1% better than using AmB alone (0.7 mg/L Pcz + 4 mg/L AmB).

The determination of the FICI [26,27] values (Figure IV.3.2B), showed that the interaction of this drug combinations were, in the majority of the cases, indifferent (>1 FICI ≥ 4), endorsing again the obtained results.

A	AmB					
Pcz	mg/L	4	2	0,5	0,25	0
	200	21,17	**17,11**	**16,14**	25,42	29,09
	100	**16,19**	**18,99**	23,58	31,75	33,66
	50	**19,36**	24,08	29,62	40,39	52,97
	0,7	**15,09**	**17,38**	46,17	67,93	44,00
	0	**16,01**	27,26	41,08	53,59	

Bold: metabolic activity below 20%.

B	AmB			
Pcz	mg/L	4	2	0,5	0,25
	200	2,26	1,18	0,98	1,36
	100	1,56	1,56	1,29	1,53
	50	1,73	1,49	1,31	1,55
	0,7	1,35	1,07	2,26	2,86

	FICI ≤ 0.5	*Synergy*
	> 0.5 FICI ≤ 1	*Additive*
	>1 FICI ≤ 4	*Indifferent*
	FICI > 4	*Antagonism*

Figure IV.3.2. Checkerboard of the combinatory effect of different concentrations of Pcz and AmB. The values are related to the metabolic activity of the biofilms, determined through the XTT assay (A). Calculated FICI range of checkerboard experiments for *C. glabrata* ATCC2001 (B).

Moreover, none of the tested combinations demonstrated a synergistic effect (FICI ≤0.5), nor an antagonistic one (FICI > 4) and only two combinations showed a slight additive effect (>0.5 FICI ≤1): 0.7 mg/L Pcz + 2 mg/L AmB (FICI= 1.07 – Additive/Indifferent) and 200 mg/L Pcz + 0.5 mg/L AmB (FICI =0.98 - Additive). In order to verify the biofilm cell and the biomass eliminations, CFU count and crystal violet were performed for the combination with the better FICI additive effect (200 mg/L Pcz + 0.5 mg/L AmB) (Figure IV.3.3). It was noted that, in both combinations, one of the antifungals had to be equal or very near the to the MBEC value to have an additive effect. This fact seems probably not clinically helpful, since it would also be interesting to low the high concentrations needed to eradicate the biofilm, when using two drugs, in order to decrease possible side effects [3].

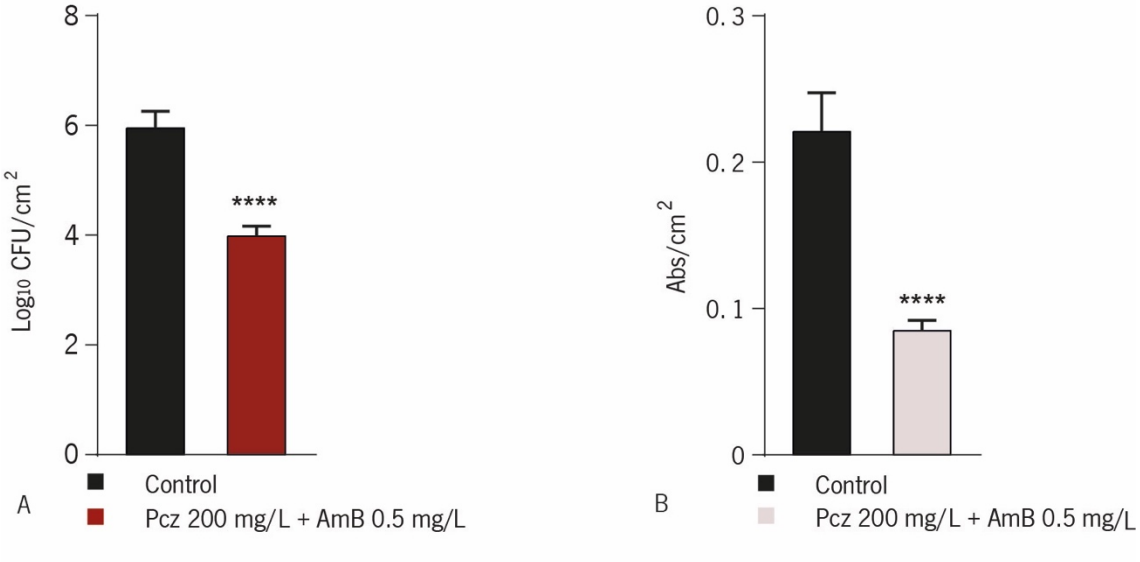


Figure IV.3.3. Effect of association of posaconazole and amphotericin B (200 + 0.5 mg/L) on *C. glabrata* ATCC2001 biofilms. Mean values of the logarithm of colony forming units normalized by unit of area (Log_{10} CFU/cm^2) (A). Crystal Violet in a 48-hour-biofilm of *C. glabrata* ATCC2001, with and without association of posaconazole and amphotericin B (200 + 0.5 mg/L) (B). The quantification of the biomass is presented by Abs/cm^2. *(**** $P< 0.0001$).*

It was expected that applying this specific association, the reduction on biofilm cells were at least 2 Log_{10} CFU/cm^2 and also that the biofilm biomass would diminish. Nevertheless, none of the objectives were properly achieved. The combination permitted a reduction of 1.49 Log_{10} CFU/cm^2 (P<0.0001), which was better result than the use of Pcz alone but did not show any clear advantages that the single use of 4 mg/L of AmB, since, in this case, the Log_{10} CFU/cm^2 reduction was higher (1.49 in combination vs 2.9 AmB alone). In fact, comparing both drugs individually, regarding the results of the CFU counts, Pcz did not reveal a pronounced effect on eradicating biofilm cells (i.e. ≥ 2 Log_{10} CFU/cm^2), applying the minimum or even the maximum dose (Figure IV.3.1 A and B).

On the other side, the good performance of the AmB alone (Figure IV.3.1B and D), demonstrated that, even though cases of tolerance and resistance have been appearing [11,12,42], AmB is still among the most effective drugs for the treatment of *Candida* spp. infections. Other authors have stated similar results in other *Candida* spp. [43–45]. Regarding the biomass reduction, similar results were obtained. The concomitant use of Pcz/AmB lead to a good biomass reduction of 69.3%, which is an important feature of an antifungal drug in a treatment of an infection caused by biofilms, yet, a higher reduction was observed when Pcz was used alone (77.6%), but lower than when AmB was used isolated (55.2% for 0.5 mg/L, data not shown) (Figure IV.3.1B and Figure

IV.3.1D). It is documented that the biofilm structure creates an exceptionally resistant profile to the antifungal drugs [2]. All these results validate that the biofilm cells are much more resistant than the planktonic and are particularly resistant to azoles (as Pcz), which has also been verified by other authors [13,46–53].

In line with our outcomes, Cacciapuoti and collegues tested several combinations of AmB/Pcz in different *C. albicans* strains and the results revealed cases of synergy but also cases of indifference [35]. Rex and colleagues showed comparable results with AmB and Flu [36]. The authors demonstrated that the combination AmB/Flu was as effective as higher-dose Flu given alone for patients with candidaemia[36]. In opposition, using other drugs combinations (AmB, flucytosine, Pcz, caspofungin or FK506), the authors also reported *in vitro* synergism [3,37]. Also, no antagonism was detected in Flu/AmB, saperconazole/AmB or SCH39304(another azole)/AmB association in candidiasis studies in murine models [38–41]. One important feature is that none of these studies were developed with biofilm cells as the present study.

As the azoles block the ergosterol production which are the target of polyenes, this can reduce the antifungal capacity of the latter (by decreasing the target concentration), which was also described in few reports[54–56]. Indeed, one of the most important mechanisms of antifungal resistance is the modulated expression of drug targets, as the membrane sterol composition of biofilms' cells. It is documented that cells from mature biofilms contain a significantly lower concentration of ergosterol, especially during the later phases of biofilm growth, compared to the planktonic cells[57–61]. Additionally, the azole resistance profile of biofilms of *C. glabrata* and the interference of the antifungals mechanisms of action explained above, may be the reasons for the less expressive results in this Pcz/AmB association, comparing to the use of the drugs alone.

Combinational drug therapies appeared due to the increase of the cases of antifungal drug resistance and are usually employed to treat patients with several immunosuppressing diseases (e.g. HIV, cancer). These approaches seem to have great potential in fungal infections therapy but require a constant evaluation of the efficacy of the proposed schemes.

References

1. Silva, S.; Rodrigues, C.F.; Araújo, D.; Rodrigues, M.E.; Henriques, M. Candida Species Biofilms' Antifungal Resistance. *J. Fungi* **2017**, *3*, 8, doi:10.3390/jof3010008.
2. Rodrigues, C. F.; Rodrigues, M. E.; Silva, S.; Henriques, M. Candida glabrata Biofilms: How Far Have We Come? *J. Fungi* **2017**, *3*, 11, doi:10.3390/JOF3010011.
3. Pappas, P. G.; Kauffman, C. A.; Andes, D. R.; Clancy, C. J.; Marr, K. A.; Ostrosky-Zeichner, L.; Reboli, A. C.; Schuster, M. G.; Vazquez, J. A.; Walsh, T. J.; Zaoutis, T. E.; Sobel, J. D. Clinical Practice Guideline for the Management of Candidiasis: 2016 Update by the Infectious Diseases Society of America. *Clin. Infect. Dis.* **2015**, *62*, e1–e50, doi:10.1093/cid/civ933.
4. Pfaller, M. A.; Diekema, D. J. Epidemiology of invasive candidiasis: A persistent public health problem. *Clin.*

Microbiol. Rev. **2007**, *20*, 133–163, doi:10.1128/CMR.00029-06.
5. Pappas, P. G. Invasive candidiasis. *Infect. Dis. Clin. North Am.* **2006**, *20*, 485–506, doi:10.1016/j.idc.2006.07.004.
6. Rodrigues, C. F.; Silva, S.; Henriques, M. Candida glabrata: A review of its features and resistance. *Eur. J. Clin. Microbiol. Infect. Dis.* **2014**, *33*, 673–688, doi:10.1007/s10096-013-2009-3.
7. Douglas, L. J. Candida biofilms and their role in infection. *Trends Microbiol.* **2003**, *11*, 30–6.
8. Costerton, J. W.; Lewandowski, Z.; Caldwell, D. E.; Korber, D. R.; Lappin-Scott, H. M. Microbial Biofilms. *Annu. Rev. Microbiol.* **1995**, *49*, 711–745, doi:10.1146/annurev.mi.49.100195.003431.
9. Tscherner, M.; Schwarzmüller, T.; Kuchler, K. Pathogenesis and Antifungal Drug Resistance of the Human Fungal Pathogen Candida glabrata. *Pharmaceuticals* **2011**, *4*, 169–186, doi:10.3390/ph4010169.
10. Henry, K.; Nickels, J.; Edlind, T. Upregulation of ERG genes in Candida species by azoles and other sterol biosynthesis inhibitors. *Antimicrob Agents Chemother.* **2000**, *44*, 2693–700.
11. Vandeputte, P.; Tronchin, G.; Larcher, G.; Ernoult, E.; Bergès, T.; Chabasse, D.; Bouchara, J.-P. A nonsense mutation in the ERG6 gene leads to reduced susceptibility to polyenes in a clinical isolate of Candida glabrata. *Antimicrob. Agents Chemother.* **2008**, *52*, 3701–9, doi:10.1128/AAC.00423-08.
12. Vandeputte, P.; Tronchin, G.; Bergès, T.; Hennequin, C.; Chabasse, D.; Bouchara, J.-P. Reduced susceptibility to polyenes associated with a missense mutation in the ERG6 gene in a clinical isolate of Candida glabrata with pseudohyphal growth. *Antimicrob. Agents Chemother.* **2007**, *51*, 982–90, doi:10.1128/AAC.01510-06.
13. Ferrari, S.; Sanguinetti, M.; De Bernardis, F.; Torelli, R.; Posteraro, B.; Vandeputte, P.; Sanglard, D. Loss of mitochondrial functions associated with azole resistance in Candida glabrata results in enhanced virulence in mice. *Antimicrob. Agents Chemother.* **2011**, *55*, 1852–60, doi:10.1128/AAC.01271-10.
14. Prażyńska, M.; Gospodarek, E. In vitro effect of amphotericin B on Candida albicans, Candida glabrata and Candida parapsilosis biofilm formation. *Mycopathologia* **2014**, *177*, 19–27, doi:10.1007/s11046-014-9727-7.
15. Choi, H. W.; Shin, J. H.; Jung, S. I.; Park, K. H.; Cho, D.; Kee, S. J.; Shin, M. G.; Suh, S. P.; Ryang, D. W. Species-specific differences in the susceptibilities of biofilms formed by Candida bloodstream isolates to echinocandin antifungals. *Antimicrob. Agents Chemother.* **2007**, *51*, 1520–1523, doi:10.1128/AAC.01141-06.
16. Colombo, A. L.; Nucci, M.; Park, B. J.; Nouér, S. A.; Arthington-Skaggs, B.; da Matta, D. A.; Warnock, D.; Morgan, J.; Brazilian Network Candidemia Study, for the B. N. C. Epidemiology of candidemia in Brazil: a nationwide sentinel surveillance of candidemia in eleven medical centers. *J. Clin. Microbiol.* **2006**, *44*, 2816–23, doi:10.1128/JCM.00773-06.
17. Zarei Mahmoudabadi, A.; Rezaei-Matehkolaei, A.; Ghanavati, F. The Susceptibility Patterns of Candida Species Isolated From Urine Samples to Posaconazole and Caspofungin. *Jundishapur J. Microbiol.* **2015**, *8*, doi:10.5812/jjm.24298.
18. Arendrup, M. C.; Prakash, A.; Meletiadis, J.; Sharma, C.; Chowdhary, A. *Candida auris*: Comparison of the EUCAST and CLSI reference microdilution MICs for eight antifungal compounds and associated tentative ECOFFs. *Antimicrob. Agents Chemother.* **2017**, doi:10.1128/AAC.00485-17.
19. Vuichard, D.; Weisser, M.; Orasch, C.; Frei, R.; Heim, D.; Passweg, J. R.; Widmer, A. F. Weekly use of fluconazole as prophylaxis in haematological patients at risk for invasive candidiasis. *BMC Infect. Dis.* **2014**, *14*, 573, doi:10.1186/s12879-014-0573-5.
20. Groll, A. H.; Walsh, T. J. Antifungal efficacy and pharmacodynamics of posaconazole in experimental models of invasive fungal infections. *Mycoses* **2006**, *49 Suppl 1*, 7–16, doi:10.1111/j.1439-0507.2006.01296.x.
21. Skiest, D. J.; Vazquez, J. A.; Anstead, G. M.; Graybill, J. R.; Reynes, J.; Ward, D.; Hare, R.; Boparai, N.; Isaacs, R. Posaconazole for the Treatment of Azole-Refractory Oropharyngeal and Esophageal Candidiasis in Subjects with HIV Infection. *Clin. Infect. Dis.* **2007**, *44*, 607–614, doi:10.1086/511039.
22. Stephens, N.; Rawlings, B.; Caffrey, P. Streptomyces nodosus host strains optimized for polyene glycosylation engineering. *Biosci Biotechnol Biochem* **2012**, *76*, 384–387, doi:10.1271/bbb.110673.
23. Storm, G.; van Etten, E. Biopharmaceutical aspects of lipid formulations of amphotericin B. *Eur J Clin Microbiol Infect Dis* **1997**, *16*, 64–73.
24. Rodrigues, C. F.; Gonçalves, B.; Rodrigues, M. E.; Silva, S.; Azeredo, J.; Henriques, M. The Effectiveness of Voriconazole in Therapy of Candida glabrata's Biofilms Oral Infections and Its Influence on the Matrix Composition and Gene Expression. *Mycopathologia* **2017**, *182*, 653–664, doi:10.1007/s11046-017-0135-7.
25. Pierce, C. G.; Uppuluri, P.; Tristan, A. R.; Wormley, F. L.; Mowat, E.; Ramage, G.; Lopez-Ribot, J. L. A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing. *Nat. Protoc.* **2008**, *3*, 1494–1500, doi:10.1038/nprot.2008.141.
26. Moody, J. Synergism testing. Broth microdilution checkerboard and broth microdilution methods. In *Clinical Microbiology Procedures Handbook*; Isenberg, H., Ed.; American Society for Microbiology: Washington, DC, 1991; pp. 1–28.
27. Sopirala, M. M.; Mangino, J. E.; Gebreyes, W. A.; Biller, B.; Bannerman, T.; Balada-Llasat, J.-M.; Pancholi, P.

Synergy testing by Etest, microdilution checkerboard, and time-kill methods for pan-drug-resistant Acinetobacter baumannii. *Antimicrob. Agents Chemother.* **2010**, *54*, 4678–83, doi:10.1128/AAC.00497-10.
28. Cataldi, V.; Di Campli, E.; Fazii, P.; Traini, T.; Cellini, L.; Di Giulio, M. *Candida* species isolated from different body sites and their antifungal susceptibility pattern: Cross-analysis of *Candida albicans* and *Candida glabrata* biofilms. *Med. Mycol.* **2016**, myw126, doi:10.1093/mmy/myw126.
29. Pfaller, M. A.; Castanheira, M. Nosocomial candidiasis: Antifungal stewardship and the importance of rapid diagnosis. *Med. Mycol.* 2016, *54*.
30. EUCAST *Breakpoint tables for interpretation of MICs, Version 8.1, valid from 2017-03-01*;
31. Pfaller, M. .; Messer, S. .; Boyken, L.; Hollis, R. .; Rice, C.; Tendolkar, S.; Diekema, D. . In vitro activities of voriconazole, posaconazole, and fluconazole against 4,169 clinical isolates of Candida spp. and Cryptococcus neoformans collected during 2001 and 2002 in the ARTEMIS global antifungal surveillance program. *Diagn. Microbiol. Infect. Dis.* **2004**, *48*, 201–205, doi:10.1016/j.diagmicrobio.2003.09.008.
32. Lemke, A.; Kiderlen, A. F.; Kayser, O. Amphotericin B. *Appl. Microbiol. Biotechnol.* **2005**, *68*, 151–162, doi:10.1007/s00253-005-1955-9.
33. Baginski, M.; Czub, J. Amphotericin B and its new derivatives-mode of action. *Curr Drug Metab* **2009**, *10*, 459–69.
34. Botero, M. C.; Puentes-Herrera, M.; Cortés, J. A. Lipid formulations of amphotericin. *Rev. Chilena Infectol.* **2014**, *31*, 518–27, doi:10.4067/S0716-10182014000500002.
35. Cacciapuoti, A.; Gurnani, M.; Halpern, J.; Norris, C.; Patel, R.; Loebenberg, D. Interaction between posaconazole and amphotericin B in concomitant treatment against Candida albicans in vivo. *Antimicrob. Agents Chemother.* **2005**, *49*, 638–42, doi:10.1128/AAC.49.2.638-642.2005.
36. Rex, J. H.; Pappas, P. G.; Karchmer, A. W.; Sobel, J.; Edwards, J. E.; Hadley, S.; Brass, C.; Vazquez, J. A.; Chapman, S. W.; Horowitz, H. W.; Zervos, M.; McKinsey, D.; Lee, J.; Babinchak, T.; Bradsher, R. W.; Cleary, J. D.; Cohen, D. M.; Danziger, L.; Goldman, M.; Goodman, J.; Hilton, E.; Hyslop, N. E.; Kett, D. H.; Lutz, J.; Rubin, R. H.; Scheld, W. M.; Schuster, M.; Simmons, B.; Stein, D. K.; Washburn, R. G.; Mautner, L.; Chu, T.; Panzer, H.; Rosenstein, R. B.; Booth, J.; National Institute of Allergy and Infectious Diseases Mycoses Study Group A Randomized and Blinded Multicenter Trial of High-Dose Fluconazole plus Placebo versus Fluconazole plus Amphotericin B as Therapy for Candidemia and Its Consequences in Nonneutropenic Subjects. *Clin. Infect. Dis.* **2003**, *36*, 1221–1228, doi:10.1086/374850.
37. Chen, Y. L.; Lehman, V. N.; Averette, A. F.; Perfect, J. R.; Heitman, J. Posaconazole Exhibits In Vitro and In Vivo Synergistic Antifungal Activity with Caspofungin or FK506 against Candida albicans. *PLoS One* **2013**, *8*, doi:10.1371/journal.pone.0057672.
38. Sanati, H.; Ramos, C. F.; Bayer, A. S.; Ghannoum, M. A. Combination therapy with amphotericin B and fluconazole against invasive candidiasis in neutropenic-mouse and infective-endocarditis rabbit models. *Antimicrob. Agents Chemother.* **1997**, *41*, 1345–8.
39. Sugar, A. M.; Hitchcock, C. A.; Troke, P. F.; Picard, M. Combination Therapy of Murine Invasive Candidiasis with Fluconazole and Amphotericin B. *Antimicrob. Agents Chemother.* **1995**, *39*, 598–601.
40. Sugar, A. M.; Salibian, M.; Goldani, L. Z. Saperconazole Therapy of Murine Disseminated Candidiasis: Efficacy and Interactions with Amphotericin B. *Antimicrob Agents Chemother* **1994**, *38*, 371–373.
41. Sugar, A. M. Interactions of Amphotericin B and SCH 39304 in the Treatment of Experimental Murine Candidiasis: Lack of Antagonism of a Polyene-Azole Combination. *Antimicrob. Agents Chemother.* **1991**, *35*, 1669–1671.
42. Canuto, M. M.; Rodero, F. G. Antifungal drug resistance to azoles and polyenes. *Lancet Infect. Dis.* **2002**, *2*, 550–563, doi:10.1016/S1473-3099(02)00371-7.
43. Pierce, C. G.; Srinivasan, A.; Uppuluri, P.; Ramasubramanian, A. K.; López-Ribot, J. L. Antifungal therapy with an emphasis on biofilms. *Curr. Opin. Pharmacol.* **2013**, *13*, doi:10.1016/j.coph.2013.08.008.
44. Delattin, N.; Cammue, B. P. a; Thevissen, K. Reactive oxygen species-inducing antifungal agents and their activity against fungal biofilms. *Future Med. Chem.* **2014**, doi:10.4155/fmc.13.189.
45. Rodrigues, C. F.; Silva, S.; Azeredo, J.; Henriques, M. Candida glabrata's recurrent infections: biofilm formation during Amphotericin B treatment. *Lett. Appl. Microbiol.* **2016**, *63*, 77–81, doi:10.1111/lam.12600.
46. Al-fattani, M. a; Douglas, L. J. Penetration of Candida Biofilms by Antifungal Agents. *Antimicrob Agents Chemother* **2004**, *48*, 3291–3297, doi:10.1128/AAC.48.9.3291.
47. De Luca, C.; Guglielminetti, M.; Ferrario, A.; Calabrò, M.; Casari, E. Candidemia: species involved, virulence factors and antimycotic susceptibility. *New Microbiol.* **2012**, *35*, 459–468.
48. Grandesso, S.; Sapino, B.; Mazzuccato, S.; Solinas, M.; Bedin, M.; D'Angelo, M.; Gion, M. Study on in vitro susceptibility of Candida spp. isolated from blood culture. *Infect Med.* **2012**, *20*, 25–30.
49. Lewis, R.; Kontoyiannis, D.; Darouiche, R.; Raad, I.; Prince, R. Antifungal activity of amphotericin B, fluconazole, and voriconazole in an in vitro model of Candida catheter-related bloodstream infection. *Antimicrob Agents Chemother.*

2002, *46*, 3499–505.
50. Donlan, R.; Costerton, J. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.* **2002**, *15*, 167–193.
51. Osawa, K.; Shigemura, K.; Yoshida, H.; Fujisawa, M.; Arakawa, S. Candida urinary tract infection and Candida species susceptibilities to antifungal agents. *J Antibiot (Tokyo).* **2013**, *66*, 651–4.
52. Narita, A.; Muramatsu, H.; Sakaguchi, H.; Doisaki, S.; et al Correlation of CYP2C19 Phenotype With Voriconazole Plasma Concentration in Children. *J Pediatr Hematol Oncol.* **2013**, *35*, (5):e21.
53. Caira, M.; Alkhamis, K.; Obaidat, R. Preparation and crystal characterization of a polymorph, a monohydrate, and an ethyl acetate solvate of the antifungal fluconazole. *J. Pharm. Sci.* **2004**, *93*, 601–61.
54. Louie, A.; Liu, W.; Miller, D. A.; Sucke, A. C.; Liu, Q.-F.; Drusano, G. L.; Mayers, M.; Miller, M. H. Efficacies of High-Dose Fluconazole plus Amphotericin B and High-Dose Fluconazole plus 5-Fluorocytosine versus Amphotericin B, Fluconazole, and 5-Fluorocytosine Monotherapies in Treatment of Experimental Endocarditis, Endophthalmitis, and Pyelonephritis D. *Antim Agents Chemoter.* **1999**, *43*, 2831–2840.
55. Lewis, R. E.; Lund, B. C.; Klepser, M. E.; Ernst, E. J.; Pfaller, M. A. Assessment of antifungal activities of fluconazole and amphotericin B administered alone and in combination against Candida albicans by using a dynamic in vitro mycotic infection model. *Antimicrob. Agents Chemother.* **1998**, *42*, 1382–6.
56. Sugar, A. M.; Liu, X. P. Interactions of itraconazole with amphotericin B in the treatment of murine invasive candidiasis. *J. Infect. Dis.* **1998**, *177*, 1660–3.
57. Santos, A.; De Mello, T.; De Souza Ramos, L.; Branquinha, M. Biofilm : A Robust and Efficient Barrier to Antifungal Chemotherapy. *J. Antimicrob. Agents* **2015**, *1*, 1–4, doi:10.4172/Antimicro.1000e101.
58. LaFleur, M. D.; Kumamoto, C. A.; Lewis, K. Candida albicans biofilms produce antifungal-tolerant persister cells. *Antimicrob. Agents Chemother.* **2006**, *50*, 3839–3846, doi:10.1128/AAC.00684-06.
59. Perlin, D. S.; Shor, E.; Zhao, Y. Update on Antifungal Drug Resistance. *Curr. Clin. Microbiol. Reports* **2015**, *2*, 84–95, doi:10.1007/s40588-015-0015-1.
60. Bonhomme, J.; d'Enfert, C. Candida albicans biofilms: Building a heterogeneous, drug-tolerant environment. *Curr. Opin. Microbiol.* 2013, *16*.
61. Mukherjee, P. K.; Chandra, J.; Kuhn, D. M.; Ghan- noum, M. A. Mechanism of fluconazole resistance in Candida albicans biofilms: phase-specific role of efflux pumps and membrane sterols. *Infect. Immun.* **2003**, *71*, 4333–4340.

V

Chapter V

Candida glabrata biofilms response to echinocandins

The work presented in this chapter was adapted from:

Rodrigues, C.F.; Rodrigues, M.E.; Henriques, M. Susceptibility of *Candida glabrata* biofilms to echinocandins: alterations on the matrix composition. *Biofouling*, **2018**, 25:1-10. doi: 10.1080/08927014.2018.

V

Susceptibility of *Candida glabrata* biofilms to echinocandins: alterations on the matrix composition

ABSTRACT

Aim: to determine the susceptibility of *C. glabrata* biofilms to echinocandins and to evaluate their effect on the biofilms' matrix composition.

Materials & Methods: drug susceptibilities were assessed through the determination of MIC, MFC and MBEC of caspofungin (Csf) and micafugin (Mcf). The content in β-1,3-glucans and proteins were assessed in the matrices, after the drugs contact and the results compared with reference strains of other *Candida* spp.

Results and Conclusions: generally, the concentration of β-1,3-glucans decreased and the proteins varied, after the contact with echinocandins. These adjustments in the matrices composition of *C. glabrata* and other *Candida* spp. biofilms, and the chemical differences between Csf and Mcf, seem responsible and may determine the effectivity in the drug responses.

Keywords: *Candida glabrata*, echinocandin, biofilm, matrix, antifungal drug, resistance.

V.1. Introduction

Infections caused by *Candida* genus (candidiasis) have been growing in the last decades and becoming more difficult to eradicate. Not less than 15 different *Candida* spp. cause infections in humans, and *Candida glabrata* is one of the most common [1,2]. These infectious are generally due to the unbalanced use of immunosuppressive drugs and broad spectrum antibiotics, the growth of immunogenic diseases, the upsurge of endocrine disorders, the widespread use of indwelling medical devices, the aging and the increase in patient's population [3–6]. Each *Candida* spp. has distinctive virulence factors, antifungal susceptibilities, and defined epidemiologies [2], but the aptitude of these organisms to form biofilms is a very particular virulence feature that allows tissue attachment, following infection of host [7]. Biofilms are communities of microorganisms embedded in an extracellular matrix [8,9], which confer significant resistance to antifungal therapy and intense host immune responses [10,11]. This matrix is composed of exopolymeric compounds secreted by sessile cells, with all classes of macromolecules providing protection against environmental challenges [12]. Infections caused by biofilms are complicated due to inducible gene networks encoding different proteins that confer tolerance or resistance to many of the available antifungal drugs [13].

Chemically, echinocandins are cyclic lipo-hexapeptides with modified N-linked acyl lipid side chains [14], biosynthesized by diverse Ascomycota fungi on non-ribosomal peptide synthase complexes [15]. The first echinocandin with antimycotic activity was discovered in the 1970s, and afterwards, over 20 natural echinocandins were isolated [15]. By disturbing the fungal cell wall synthesis through a non-competitive inhibition of β-1,3-glucan synthesis, these drugs weaken the cell wall, break down the cellular integrity and, finally, induce cell lysis [16,17]. Due to this mechanism of action the echinocandins (which include anidulafungin, caspofungin, and micafungin) are generally well tolerated, avoiding the over-lapping toxicities and drug-drug interactions with mammalian cells which are observed with the azoles and the polyenes [18,19]. Also, they are safe to use and are associated with few adverse reactions and few drug-drug interactions of significance [14]. Presently, because of their high clinical efficacy in the non-neutropenic patient population, in patients with moderately severe to severe illness, and in patients with pre-azole exposure [2,20], the established protocols recommend echinocandins as first-line antifungal agents to treat invasive candidiasis, especially *C. glabrata* due to its innate high azole resistance [2,21]. Though these antifungal drugs are active against most important *Candida* spp., in which they display *in vitro* fungicidal activity [22], in critically ill patients, it is recognised that the achievement of their

pharmacodynamic and pharmacokinetic targets show a large inter-individual variability [14]. In Europe, micafungin (Mcf) is approved for use in paediatric patients of any age including neonates, while caspofungin (Csf) is approved for use in paediatric patients ≥1 year of age, since there are insufficient data regarding its use in those <1 year of age [23,24]. Csf and Mcf use is limited by their necessity for once-daily intravenous dosage regimen, lack of oral formulation and limited spectrum [2,22,25], but both echinocandins still show very good *in vitro activity* against clinically relevant isolates of *Candida* spp. [26–30]. In trials involving adult and paediatric patients with invasive and oesophageal candidiasis, Mcf showed to be non-inferior to intravenous Csf, intravenous fluconazole or liposomal amphotericin B. The tolerability profile of Csf and Mcf are, in general, similar to fluconazole and are better tolerated than liposomal amphotericin B or oral itraconazole [31].

The goal of this work was to evaluate seven *C. glabrata* clinical isolates, by comparison with *C. albicans*, *C. parapsilosis* and *C. tropicalis*, regarding their susceptibility to Csf and Mcf and the biochemical variations induced on the biofilm matrices' composition after the drug exposure.

V.2. Material and Methods

V.2.1 Organisms

A total of ten strains were used in the course of this study. Six clinical isolates of *C. glabrata* from Hospital Escala Braga in Portugal, recovered from different sites: oral cavity (*C. glabrata* AE2 and D1), urinary tract (*C. glabrata* 562123 and 513100), vaginal tract (*C. glabrata* 534784 and 585626), and four reference strains from the American Type Culture Collection (*C. glabrata* ATCC2001, *C. albicans* SC5314, *C. parapsilosis* ATCC22019 and *C. tropicalis* ATCC750). The identity of all isolates was confirmed using CHROMagar™ *Candida* (CHROMagar™, Paris, France) and by PCR-based sequencing using specific primers (*ITS1* and *ITS4*) against the 5.8s subunit gene reference. Genomic DNA was extracted following previously described procedures [32]. The PCR products were sequenced using the ABI-PRISM Big Dye terminator cycle sequencing kit (Perkin Elmer, Applied Biosystems, Warrington, UK).

V.2.2 Growth conditions

For each experiment, strains were subcultured on Sabouraud dextrose agar (SDA) (Merck, Darmstadt, Germany) for 24 h at 37°C. Cells were then inoculated in Sabouraud dextrose broth (SDB) (Merck, Darmstadt, Germany) and incubated for 18 h at 37°C, under agitation at 120 rpm.

After incubation, the cells were harvested by centrifugation, 3000 *g* for 10 min, at 4°C and washed twice with Phosphate Buffer Saline (PBS 0.1 *M*, pH=7.5). Pellets were then suspended in RPMI-1640 (pH=7, Sigma-Aldrich, Roswell Park) and the cellular density was adjusted to $1x10^5$ cells/mL, using a Neubauer counting chamber.

V.2.3 Antifungal drugs

Csf and Mcf were kindly provided by MSD® and Astellas®, respectively. Aliquots of 5000 mg/L were prepared using dimethyl-sulfoxide (DMSO). The final concentrations used were prepared with RPMI-1640 for both drugs.

V.2.4 Antifungal susceptibility tests

All the antifungal susceptibility tests were performed using the microdilution method, in accordance to the European Committee on Antimicrobial Susceptibility Testing guidelines (EUCAST) [33,34].

V.2.5 Minimum Inhibitory Concentrations (MICs)

The inoculum was prepared by suspending five distinct colonies, ≥1 mm diameter from 24 h cultures, in at least 3 mL of sterile distilled water. Then, the inoculum was suspended by vigorous shaking on a vortex mixer for 15 s and the cell density was adjusted to the density of a 0.5 McFarland standard and adding sterile distilled water as required, giving a yeast suspension of 1-$5x10^6$ CFU/mL (colony forming units). A working suspension was prepared by a dilution of the standardised suspension in sterile distilled water to yield 1-$5x10^5$ CFU/mL. The 96-well-plate was prepared with 100 µL of cell suspension and 100 µL of each antifungal agent (Csf: 0.001 to 0.075 mg/; Mcf: 0.002 to 0.2 mg/– 2x concentrated) and incubated at 37°C, during 18-48 h. Controls without antifungal agents were also performed. Finally, the results were visualized by naked eye.

V.2.6 Minimum Fungicidal Concentration (MFC)

In addition to the previous step, 20 µL of each cell suspension treated with Csf and Mcf was recovered to a new well and serial decimal dilutions in PBS were plated onto SDA. Agar plates were incubated for 24 h at 37°C, and the total number of CFUs was determined. The results were presented as Log_{10} CFU per unit area (Log_{10} CFU/cm^2) [10].

V.2.7 Minimum Biofilm Eradication Concentration (MBEC)

Standardized cell suspensions (200 µL) were placed into selected wells of 96-wells polystyrene microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium). RPMI-1640 was used without cells, but with antifungal agent, as a negative control. As positive control cell suspensions were tested without the antifungal agent. At 24 h, 100 µL of RPMI-1640 was removed and an equal volume of fresh RPMI-1640 plus the respective antifungal concentration was added (Csf: 0.5 to 3 mg/; Mcf: 3 to 17 mg/- 2x concentrated). The plates were incubated at 37°C for more 24 h, a total of 48 h, at 120 rpm. The number of cultivable cells on biofilms was determined by the enumeration of CFUs. For that, after the period of biofilm formation, all medium was aspired and the biofilms washed once with 200 µL of PBS to remove non-adherent cells. Then, biofilms were scraped from the wells and the suspensions were vigorously vortexed for 2 min to disaggregate cells from the matrix. Serial decimal dilutions in PBS were plated on SDA and incubated for 24 h at 37°C. The results were presented as total of CFUs per unit area (Log_{10} CFU/cm^2) [35].

V.2.8 Biofilm Analysis

V.2.8.1 Biofilm total biomass quantification – Crystal Violet Staining

Total biofilm biomass was quantified by crystal violet (CV) staining [10]. After biofilm formation, the medium was aspirated and non-adherent cells removed by washing the biofilms with sterile ultra-pure water. Then, biofilms were fixed with 200 µLof methanol, which was removed after 15 min of contact. The microtiter plates were allowed to dry at room temperature, and 200 µL of CV (1% v/v) were added to each well and incubated for 5 min. The wells were then gently washed twice with sterile, ultra-pure water and 200 µL of acetic acid (33% v/v) were added to release and dissolve the stain. The absorbance of the obtained solution was read in triplicate in a microtiter plate reader (Bio-Tek Synergy HT, Izasa, Lisbon, Portugal) at 570 nm. The results were presented as absorbance per unit area (Abs/cm^2).

V.2.8.2 Biofilm structure visualization

In order to examine the structure of biofilms, after biofilm formation in the presence or absence of both drugs, they were observed by scanning electron microscopy. For that, biofilms formed as described above were dehydrated with ethanol (using 70% ethanol for 10 min, 95% ethanol for 10 min and 100% ethanol for 20 min) and air dried for 20 min. Samples were kept in a desiccator until further analysis. Prior to observation, the base of the wells was mounted onto aluminum stubs,

sputter coated with gold and observed with an S-360 scanning electron microscope (Leo, Cambridge, USA).

V.2.8.3 Biofilm's matrix composition evaluation

Extraction method: biofilms were formed in 24-well polystyrene microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium) [35]. For this, 1000 µL of yeast cell suspension ($1x10^5$ cells/mL in RPMI-1640 or RPMI-1640 with Csf and Mcf at concentration corresponding to the MBEC of each species/strain) were added to each well and the biofilms were performed as described previously. After 24 h, 500 µL of RPMI-1640 medium was removed and an equal volume of fresh RPMI-1640 with or without the antifungal agents was added. After 48 h, biofilms were scraped from the 24-well plates, resuspended in ultra-pure water, sonicated (Ultrasonic Processor, Cole-Parmer, Illinois, USA) for 30 s at 30 W, and then the suspension vortexed for 2 min. The suspension was centrifuged at 5000 *g* for 5 min at 4°C and the supernatant filtered through a 0.2 µm nitrocellulose filter. The pellets were dried at 37°C until reaching a constant dry biofilm weight.

V.2.8.4 β-1,3-glucans concentration determination

The β-1,3-glucans concentrations were determined using Glucatell® kit (Cape Cod®, East Falmouth, USA). The values were normalized per pg/µg of β-1,3-glucans/total of polysaccharides content (evaluated by the procedure proposed by Dubois et al [36]).

V.2.8.5 Protein concentration determination

The protein content of the biofilm matrix was measured using the BCA® Kit (Bicinchoninic Acid, Sigma-Aldrich, St Louis, USA), using bovine serum albumin (BSA) as a standard. The values were normalized per g of dry weight of biofilm and presented as mg of protein per g of dry weight of biofilm.

V.2.9 Statistical Analysis

All these experiments were repeated three times in at least three independent assays. Results were compared using one-way and two-way ANOVA, Tukey's and Dunnett's post hoc multiple comparisons tests, using GraphPad™ Prism 7™ (CA, USA) software. All tests were performed with a confidence level of 95%

V.3. Results

V.3.1 Planktonic and biofilm susceptibility to Csf and Mcf

Table V.1 shows the MIC, MFC and MBEC determined for all the strains used in this study. It is possible to observe that these values were species/strain dependent, but, in general, MFC and MBEC of Mcf were higher than those from Csf. This behaviour is specially noticed in the concentration needed to eradicate the biofilm (MBEC). In those cases, the concentrations were, sometimes 5 to 6 times higher than the MFC. Mostly, in biofilm form (MBEC), all *C. glabrata* strains demonstrated to have similar resistant profiles to *C. albicans* SC5314, *C. tropicalis* ATCC750 and *C. parapsilosis* ATCC22019.

V.3.2 Biofilm reduction capacity of Csf and Mcf

Crystal violet staining was used to evaluate the biomass reduction of the *Candida* spp. biofilms, after a 24h of contact with Csf or Mcf (Table V.2). Although both agents have a good capacity in reducing the biomass, Csf had a higher capacity than Mcf. Interestingly, the lowest percentage of biomass reduction for Csf and Mcf was obtained for four of the seven *C. glabrata* strains (585626, 534784, D1 and ATCC2001), demonstrating the resistance profile of this species, comparing to the other species of *Candida* spp.. On the other side, *C. albicans* SC5314 ($P<0.001$ for Csf and Mcf) and *C. tropicalis* ATCC750 ($P<0.001$ for Csf and $P<0.05$ for Mcf) demonstrated to have the most expressive biomass' reductions.

V.3.3 Biofilm structure

SEM images (Figure V.1A and V.1B) confirmed that all *Candida* spp. had good capacity for biofilm production, specially *C. glabrata* AE2, *C. glabrata* D1 (biofilms formed by yeasts in a long continuous carpet [3,10,37]), *C. albicans* SC5314 (biofilm presenting high hyphae quantity and entanglement [3,38]) and *C. parapsilosis* ATCC22019 (continuous biofilm carpet with clumped blastospores [3,39]). *C. tropicalis* ATCC750 biofilm can be described as chains of cells with high amounts of extracellular material [3,40,41]. After Csf and Mcf contact, the SEM images confirmed the crystal violet results for all species/strains (Figure V.1B). It was observed a biofilm reduction in the presence of both drugs, but especially with Csf, and the biofilm cells presented a more concave aspect and appear to have a reduction in the extracellular matrix. However, the extracellular matrix of the different biofilms in the presence of the drugs seems to be different, which may be explained by the variations in β-1,3-glucans and proteins contents, as evaluated next.

Table V.1. MIC, MFC and MBEC values (mg/L) for Csf and Mcf of *C. glabrata*, *C. albicans, C. parapsilosis* and *C. tropicalis* strains

Origin	Species/Strain		Determination	Caspofungin	Micafungin
Reference	*C. glabrata*	ATCC2001	MIC	0.064	0.032
			MFC	0.10	0.20
			MBEC	**2.5-3**	**16-17**
Oral Cavity		AE2	MIC	≤0.003	0.002
			MFC	0.003	0.06
			MBEC	**0.5-1**	**7-8**
		D1	MIC	0.003	0.002
			MFC	0.003	0.06
			MBEC	**2.5-3**	**3-3.5**
Urinary Tract		562123	MIC	0.001	0.01
			MFC	0.001	0.10
			MBEC	**0.5-1**	**16-17**
		513100	MIC	0.008	0.01
			MFC	0.008	0.06
			MBEC	**2-2.5**	**16**
Vaginal Tract		534784	MIC	≤0.001	0.002
			MFC	0.05	0.06
			MBEC	**2.5-3**	**5.5-6**
		585626	MIC	0.003	0.01
			MFC	0.003	0.06
			MBEC	**2.5**	**5-5.5**
Reference	*C. albicans* SC5314		MIC	0.016	≤0.016
			MFC	0.016	0.06
			MBEC	**2.5-3**	**3.5**
Reference	*C. parapsilosis* ATCC22019		MIC	0.016	>0.032
			MFC	0.08	0.2
			MBEC	**2.5**	**16**
Reference	*C. tropicalis* ATCC750		MIC	0.008	0.002
			MFC	0.016	0.06
			MBEC	**2.5**	**16**

(Bold: MBEC values)

V.3.4 Matrices composition after Csf and Mcf contact

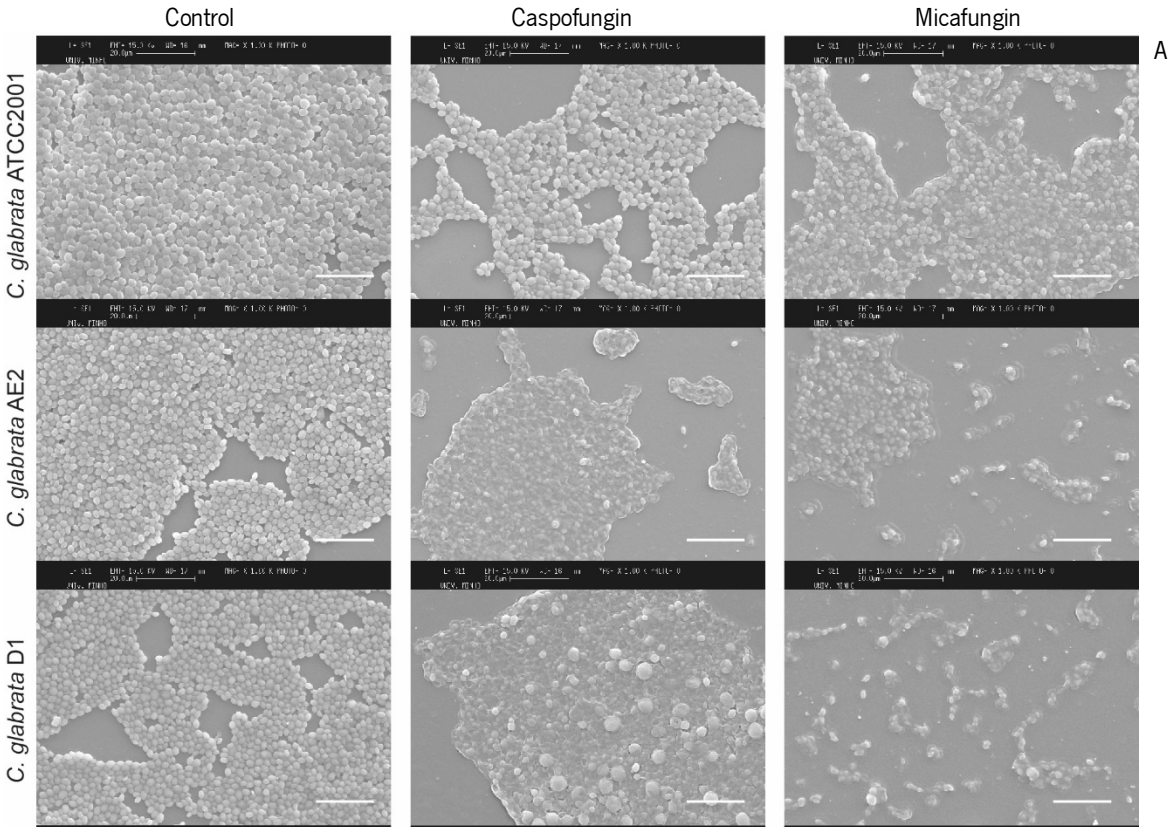
Relatively to the results from the determination of the β-1,3-glucans concentrations it can be seen that, comparing to the control groups and with some excpetions, the β-1,3-glucans generally tend to statistically decrease their presence in the biofilm matrices of all *Candida* spp., after contact with Csf and specially with Mcf (Figure V.2). This reduction was not so prominent in *C. glabrata*, when compared to the other species (Figure V.2).

Regarding the range of protein content, the profiles were dependent and very dissimilar between species/strains (Table V.3). For the majority of the species the values obtained were below the limit of detection of the BCA® Kit, which has happened before [35,42].

Table V.2. Percentage of biomass reduction on *C. glabrata*, *C. albicans, C. parapsilosis and C. tropicalis strains* after caspofungin and micafungin contact. The concentrations applied in each species/strain were the ones determined by the MBECs *(* P<0.05; ** P<0.001; *** P<0.0005; **** P< 0.0001)*

Species/Strain		% Biomass Reduction (*P* value)	
		Caspofungin	**Micafungin**
C. glabrata	ATCC2001	82.96 (**)	19.10 (*)
	AE2	79.71 (***)	39.28 (**)
	D1	69.71 (*)	23.84 (ns)
	562123	85.16 (**)	70.47 (**)
	513100	88.47 (****)	66.59 (****)
	534784	73.38 (*)	24.46 (ns)
	585626	77.85 (**)	53.63 (**)
C. albicans SC5314		92.33 (***)	82.13 (***)
C. parapsilosis ATCC22019		78.55 (***)	38.86 (**)
C. tropicalis ATCC750		84.20 (**)	53.49 (*)

(ns: non-significant)



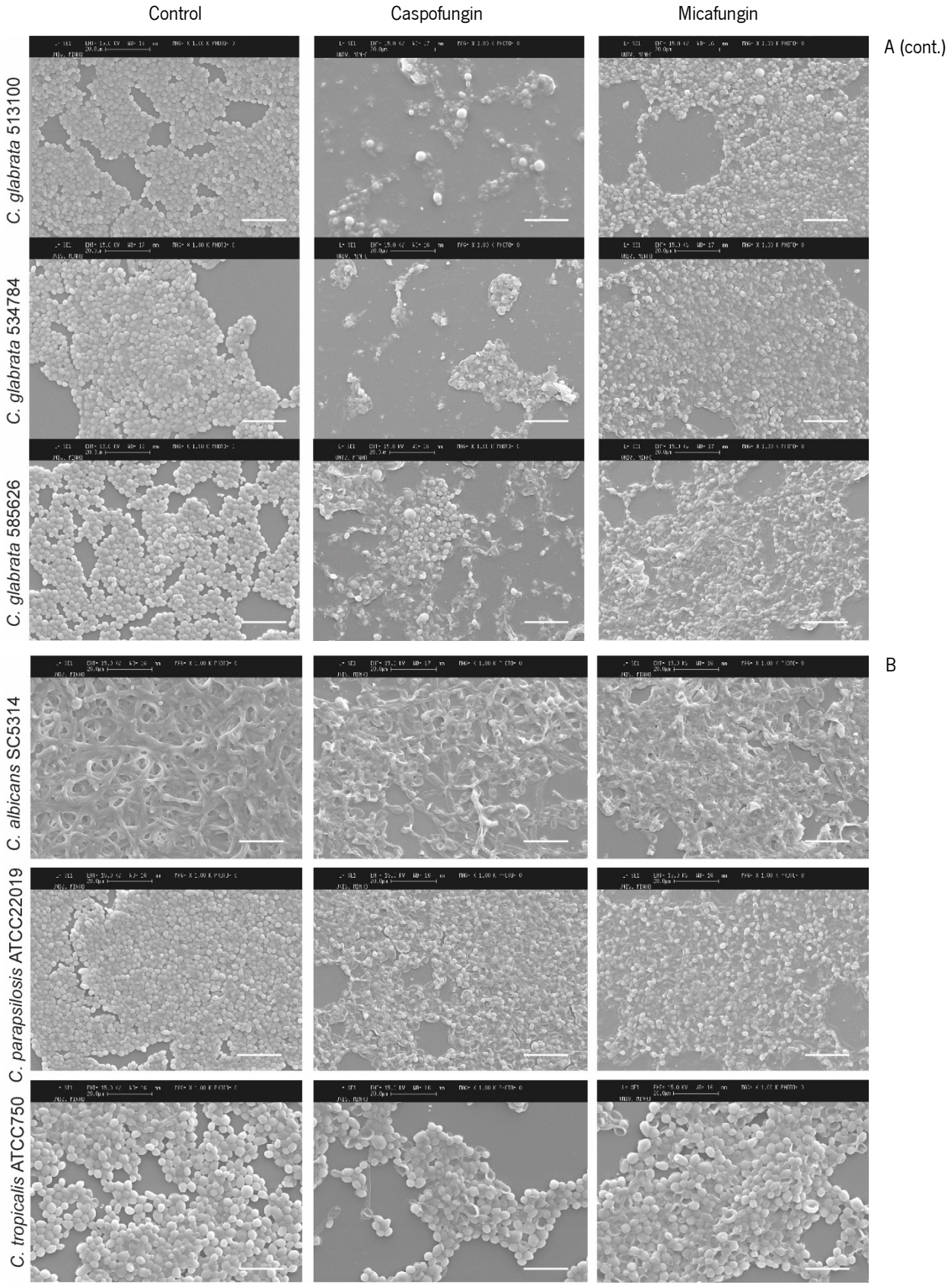


Figure V.1. SEM observations of biofilms *C. glabrata* (A), *C. albicans, C. parapsilosis and C. tropicalis* (B) strains/species grown in without drugs (control) and after caspofungin and micafungin contact. The concentrations applied in each species/strain were the ones determined by the MBECs. Magnification: 1000x. Measure bar = 20 μm.

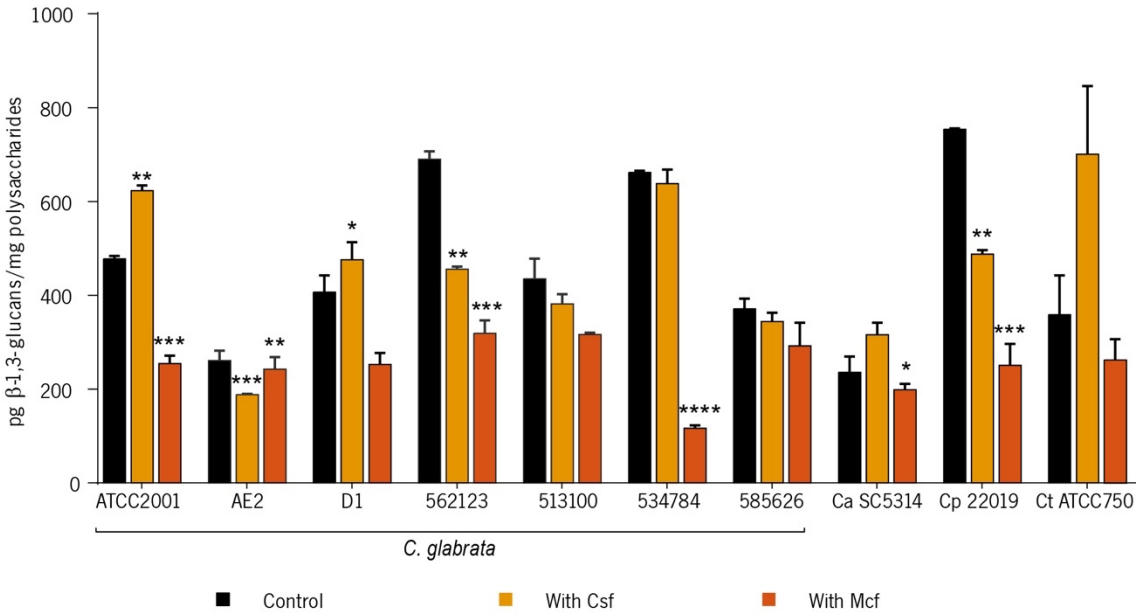


Figure V.2. β-1,3-glucans concentration/polysaccharides content (pg/μg) in 48-h-biofilm matrices of *C. glabrata*, *C. albicans* (Ca), *C. parapsilosis* (Cp) and *C. tropicalis* (Ct) strains *(* P<0.05; ** P<0.001; *** P<0.0005; **** P< 0.0001).*

V.4. Discussion

Systemic candidiasis is a growing problem in the hospitals worldwide [43,44]. With a high morbidity, it is responsible for a noteworthy mortality rate and high economic costs [3,45]. The present study evaluated seven *C. glabrata* strains and compared with reference strains of *C. albicans*, *C. parapsilosis* and *C. tropicalis,* regarding their susceptibility the most two used echinocandins – the first-line antifungal agents to treat systemic candidiasis [2,25]. Regarding MIC values of Mcf assessments, EUCAST guidelines indicate breakpoints of 0.032 mg/L for *C. glabrata*, 0.016 mg/L for *C. albicans*, 0.002-2 mg/L for *C. parapsilosis*. For *C. tropicalis,* the values are defined as 1-2 two-fold dilution steps higher than for *C. albicans* and *C. glabrata*, but EUCAST attest that there is insufficient evidence to indicate whether the wild-type population of can be considered susceptible to Mcf [34]. About the MIC Csf values, EUCAST breakpoints have not yet been established, due to significant inter-laboratory variation in the ranges for this drug [34]. In fact, there was a strong difficulty in determining these parameters, possibly due to this fact and also due to the occurrence of the paradoxical growth of the isolates of *Candida* spp. at high drug levels. This is described phenomenon, recognized as a robust mechanism of antifungal resistance, connected to an increase in chitin biosynthesis [16,20,46–51]. Nevertheless, the results confirmed that all species/strains were Mcf susceptible (Table V.1)

Table VI.3. Range of protein content (mg/g biofilm) in in 48-h-biofilm matrices of *C. glabrata*, *C. albicans, C. parapsilosis and C. tropicalis strains.* (Csf: caspofungin; Mcf: micafungin)

Species/Strain		mg protein/g biofilm
C. glabrata	*ATCC2001*	Control: 102.39 – 133.33
		Csf: BDL
		Mcf: BDL
	AE2	Control: BDL
		Csf: ≤ 12.35
		Mcf: BDL
	D1	Control: BDL
		Csf: ≤ 48.46
		Mcf: BDL
	562123	Control: 251.85 – 397. 22
		Csf: BDL
		Mcf: BDL
	513100	Control: BDL
		Csf: 187.04 – 191.96
		Mcf: BDL
	534784	Control: 34.72 – 43.59
		Csf: ≤ 9.72
		Mcf: BDL
	585626	Control: 118.31 – 156.88
		Csf: 106.11 - 106.48
		Mcf: BDL
C. albicans SC5314		Control: BDL
		Csf: 16.27 – 20.83
		Mcf: ≤ 35.80
C. parapsilosis ATCC22019		Control: BDL
		Csf: BDL
		Mcf: BDL
C. tropicalis ATCC750		Control: BDL
		Csf: BDL
		Mcf: ≤ 40.00

(BDL – Bellow the detection limit)

EUCAST guidelines also refer that isolates that are susceptible to Mcf should be considered susceptible to Csf [34], hence all species/strains were considered susceptible to this drug (Table V.1). Our results demonstrated that although Mcf concentration were, in most cases, higher than Csf, in general, the MIC, MFC and MBEC values were species/strain dependent. *C. glabrata* strains revealed to have similar MIC, MFC and MBEC profiles to *C. albicans* SC5314, *C. tropicalis* ATCC750 and *C. parapsilosis* ATCC22019. Besides, MBEC results corroborate once more the evidences that biofilm cells are more resistant to antifungal agents than planktonic cells [9,52–56]. In general, the determined *C. glabrata* MIC values showed to be close, but slightly lower than the described in the literature for some strains [31,57], which may be related to the intra-strains

variations or the described inter-laboratory and techniques variations [34]. Yet, and as the reports evidently support, *C. glabrata* and *C. parapsilosis* show higher MIC values than the other *Candida* spp. [20,46,58–61]. It is known that some *Candida* spp. – as *C. glabrata* - have naturally occurring polymorphisms in *FKS* genes, which strongly reduce their susceptibility to echinocandin drugs [29,60]. Also, it is recognized that *C. parapsilosis* family (*C. orthopsilosis* and *C. metapsilosis*) and *Candida guilliermondii*, normally, have higher MIC values comparing to other susceptible *Candida* spp. [29,62–64].

Results of the crystal violet indicated, all together, a very effective reduction on the biomass of the species/strains was achieved after the echinocandin exposure (Table V.2). However, Csf showed to have a higher biomass reduction capacity than Mcf, with a minimal reduction of 70% of the biofilms. Similar results have been shown before in studies with catheters [65–67]. On the other side, Mcf did not reveal a complete eradication of the biofilm as Csf, which has also been reported before [68,69]. This outcome was particularly noticed in *C. glabrata* (four of the seven strains had the lowest Mcf biomass percentages reduction), which can be related to the usual antifungals resistance profile of this species [3,70–72]. Curiously, the effectiveness in the biomass reduction by Csf was not always related to the use of the highest concentration, but by applying adequate concentration for a certain strain, which confirmed the detected paradoxical effect [73], described above.

In order to evaluate the extent of antifungal action of Csf and Mcf on the biofilms, SEM images were taken (Figure V.1A and V.1B). The controls revealed that all *Candida* spp. had good capacity for biofilm production. After Csf and Mcf contact (Figure V.1B), and particularly with Csf, a clear biofilm reduction, was observed. The biofilm cells seemed to be disrupted and presented a more concave aspect, endorsing the crystal violet results (Table V.2). This disruption and change in the cells configuration is expected to result from the mechanism of action of the echinocandins, namely the non-competitive inhibition of β-1,3-glucan synthesis [20,44,51] which affected the cell wall and the matrix composition. In fact, fungal cell wall polysaccharides are significant constituents of the *Candida* spp. biofilm matrix [74,75]. The biofilm matrices' composition of *C. glabrata*, *C. albicans, C. parapsilosis and C. tropicalis* strains with and without Csf and Mcf contact were evaluated in terms of β-1,3-glucans content (Figure V.2) and proteins (Table V.3). To authors' knowledge, this is the first report describing the content in biofilm matrices of *Candida* spp. in contact with these two echinocandins. The β-1,3-glucans are a group of specific polysaccharides from the *Candida* spp. cell wall that are also recognized as major constituents of the biofilm's matrices of this genus

[74,75]. After adding Csf and Mcf to the pre-formed biofilms, the fallouts from the determination of the β-1,3-glucans' concentrations (normalized per total of polysaccharides) (Figure V.2) demonstrated that, these compounds are, in general, statistically significantly probable to decline their presence in the biofilm matrices of *C. glabrata*, and in the other *Candida* spp.. Exceptions were noted only after the addition of Csf to *C. glabrata* ATCC2001, *C. glabrata* D1 and *C. tropicalis* ATCC750. It is also important to notice that the concentration of the β-1,3-glucans was very dependent on the strain and the species and that *C. glabrata* and *C. parapsilosis* showed to have the highest concentrations on the biofilm matrices, which could be a probable cause of the referred higher resistant profile, comparing to *C. albicans* (Figure V.2). Another source of variability in the drug response can be the chemical differences between the two echinocandins. Both Csf and Mcf have a cyclic peptide structure with a N-aryl group but with different patterns of hydroxylations and amino groups (R2 to R4). The N-aryl side chain (position R1) plays a critical role in the potency and toxicity and is the main point for chemical modifications of the echinocandin analogues [17,19]. Csf and Mcf have also variations in R2, R3 and R4 positions: Csf is more hydroxylated and has more amino groups, while Mcf has more aryl groups. It is documented that the replacement of the linoleoyl side chain with aryl side chains of low lipophilicity, non-linear configuration or chains switched with highly polar groups end in loss of antifungal activity, which suggests that planar, non-polar substitutions are critical for the antifungal activity [17,19]. These modifications were performed on Mcf and are the probable explanation for the differences found in this work for the studied species and strains, regarding the higher MBEC values and matrices variations, when compared to Csf.

This study showed a general parallelism in the efficacy of Csf and Mcf susceptibilities of *C. glabrata* in comparison to *C. albicans, C. parapsilosis* and *C. tropicalis* for planktonic cells (MIC/MFC). In the biofilm form and when compared to *C. albicans* and *C. tropicalis* biofilms, *C. glabrata* demonstrated to have superior resistant profiles (MBEC and matrix variations), which were similar to *C. parapsilosis*. It seems plausible to say that the external alterations in the matrices' composition and the chemical alterations on the Csf and Mcf molecules can partially explain and determine the effectiveness response to these two drugs on the biofilms infections of *Candida* spp. and, particularly, to *C. glabrata*.

References

1. Pappas, P. G. Invasive candidiasis. *Infect. Dis. Clin. North Am.* **2006**, *20*, 485–506, doi:10.1016/j.idc.2006.07.004.
2. Pappas, P. G.; Kauffman, C. A.; Andes, D. R.; Clancy, C. J.; Marr, K. A.; Ostrosky-Zeichner, L.; Reboli, A. C.; Schuster, M. G.; Vazquez, J. A.; Walsh, T. J.; Zaoutis, T. E.; Sobel, J. D. Clinical Practice Guideline for the Management of Candidiasis: 2016 Update by the Infectious Diseases Society of America. *Clin. Infect. Dis.* **2015**, *62*, e1–e50, doi:10.1093/cid/civ933.
3. Silva, S.; Rodrigues, C.; Araújo, D.; Rodrigues, M.; Henriques, M. Candida Species Biofilms' Antifungal Resistance. *J. Fungi* **2017**, *3*, 8, doi:10.3390/jof3010008.
4. Li, L.; Redding, S.; Dongari, B. Candida glabrata, an emerging oral opportunistic pathogen. *J Dent Res.* **2007**, *86*, 204 – 215 .
5. Garcia-Cuesta, C.; Sarrion-Pérez, M.-G.; Bagán, J. V; Sarrion-Perez, M. G.; Bagan, J. V Current treatment of oral candidiasis: A literature review. *J Clin Exp Dent* **2014**, *6*, e576-82, doi:10.4317/jced.51798.
6. Douglas, L. J. Candida biofilms and their role in infection. *Trends Microbiol.* **2003**, *11*, 30–6.
7. McCall, A.; Edgerton, M. Real-Time Approach to Flow Cell Imaging of Candida albicans Biofilm Development. *J. Fungi* **2017**, *3*, 13, doi:10.3390/jof3010013.
8. Costerton, J. W.; Lewandowski, Z.; Caldwell, D. E.; Korber, D. R.; Lappin-Scott, H. M. Microbial Biofilms. *Annu. Rev. Microbiol.* **1995**, *49*, 711–745, doi:10.1146/annurev.mi.49.100195.003431.
9. Donlan, R.; Costerton, J. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.* **2002**, *15*, 167–193.
10. Fonseca, E.; Silva, S.; Rodrigues, C. F.; Alves, C. T.; Azeredo, J.; Henriques, M. Effects of fluconazole on Candida glabrata biofilms and its relationship with ABC transporter gene expression. *Biofouling* **2014**, *30*, 447–57, doi:10.1080/08927014.2014.886108.
11. Rodrigues, C. F.; Silva, S.; Henriques, M. Candida glabrata: A review of its features and resistance. *Eur. J. Clin. Microbiol. Infect. Dis.* **2014**, *33*, 673–688, doi:10.1007/s10096-013-2009-3.
12. Pierce, C.; Vila, T.; Romo, J.; Montelongo-Jauregui, D.; Wall, G.; Ramasubramanian, A.; Lopez-Ribot, J. The Candida albicans Biofilm Matrix: Composition, Structure and Function. *J. Fungi* **2017**, *3*, 14, doi:10.3390/jof3010014.
13. Ramage, G.; Rajendran, R.; Sherry, L.; Williams, C. Fungal biofilm resistance. *Int. J. Microbiol.* **2012**, *2012*, 528521, doi:10.1155/2012/528521.
14. Chang, C. C.; Slavin, M. A.; Chen, S. C. A. C.-A. New developments and directions in the clinical application of the echinocandins. In *Archives of Toxicology*; 2017; Vol. 91, pp. 1613–1621.
15. Emri, T.; Majoros, L.; Tóth, V.; Pócsi, I. Echinocandins: production and applications. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 3267–84, doi:10.1007/s00253-013-4761-9.
16. Perlin, D. S.; Teppler, H.; Donowitz, G. R.; Maertens, J. A.; Baden, L. R.; al., et; Milne, S.; Brown, A. J.; Gow, N. A. Resistance to echinocandin-class antifungal drugs. *Drug Resist. Updat.* **2007**, *10*, 121–30, doi:10.1016/j.drup.2007.04.002.
17. Debono, M.; Gordee, R. S. S. Antibiotics that inhibit fungal cell wall development. *Annu. Rev. Microbiol.* **1994**, *48*, 471–497., doi:10.1146/annurev.mi.48.100194.002351.
18. Wiederhold, N. P.; Najvar, L. K.; Bocanegra, R.; Molina, D.; Olivo, M.; Graybill, J. R. In vivo efficacy of anidulafungin and caspofungin against Candida glabrata and association with in vitro potency in the presence of sera. *Antimicrob. Agents Chemother.* **2007**, *51*, 1616–1620, doi:10.1128/AAC.00105-07.
19. Wiederhold, N. P.; Lewis, R. E. The echinocandin antifungals: an overview of the pharmacology, spectrum and clinical efficacy. *Expert Opin. Investig. Drugs* **2003**, *12*, 1313–33, doi:10.1517/13543784.12.8.1313.
20. Perlin, D. S. Mechanisms of echinocandin antifungal drug resistance. *Ann N Y Acad Sci* **2015**, 1–11, doi:10.1111/nyas.12831.
21. Perlin, D. S. Resistance to echinocandin- class antifungal drugs. *Drug Resist. Updat.* **2007**, *10*, 121–130.
22. Barchiesi, F.; Spreghini, E.; Tomassetti, S.; Arzeni, D.; Giannini, D.; Scalise, G. Comparison of the Fungicidal Activities of Caspofungin and Amphotericin B against Candida glabrata. *Antimicrob. Agents Chemother.* **2005**, *49*, 4989–4992, doi:10.1128/AAC.49.12.4989-4992.2005.
23. Tragiannidis, A.; Dokos, C.; Lehrnbecher, T.; Groll, A. H. Antifungal Chemoprophylaxis in Children and Adolescents with Haematological Malignancies and Following Allogeneic Haematopoietic Stem Cell Transplantation. *Drugs* **2012**, *72*, 685–704, doi:10.2165/11599810-000000000-00000.
24. Viscoli, C.; Bassetti, M.; Castagnola, E.; Cesaro, S.; Menichetti, F.; Ratto, S.; Tascini, C.; Giacobbe, D. R. Micafungin for the treatment of proven and suspected invasive candidiasis in children and adults: findings from a multicenter prospective observational study. *BMC Infect. Dis.* **2014**, *14*, 725, doi:10.1186/s12879-014-0725-7.
25. McCarty, T. P.; Pappas, P. G. Invasive Candidiasis. *Infect. Dis. Clin. North Am.* 2016, *30*.
26. Pappas, P. G.; Rotstein, C. M. F.; Betts, R. F.; Nucci, M.; Talwar, D.; De Waele, J. J.; Vazquez, J. A.; Dupont, B.

F.; Horn, D. L.; Ostrosky-Zeichner, L.; Reboli, A. C.; Suh, B.; Digumarti, R.; Wu, C.; Kovanda, L. L.; Arnold, L. J.; Buell, D. N. Micafungin versus Caspofungin for Treatment of Candidemia and Other Forms of Invasive Candidiasis. *Clin. Infect. Dis.* **2007**, *45*, 883–893, doi:10.1086/520980.

27. Spreghini, E.; Orlando, F.; Sanguinetti, M.; Posteraro, B.; Giannini, D.; Manso, E.; Barchiesi, F. Comparative effects of micafungin, caspofungin, and anidulafungin against a difficult-to-treat fungal opportunistic pathogen, Candida glabrata. *Antimicrob. Agents Chemother.* **2012**, *56*, 1215–22, doi:10.1128/AAC.05872-11.

28. Kohno, S.; Izumikawa, K.; Yoshida, M.; Takesue, Y.; Oka, S.; Kamei, K.; Miyazaki, Y.; Yoshinari, T.; Kartsonis, N. A.; Niki, Y. A double-blind comparative study of the safety and efficacy of caspofungin versus micafungin in the treatment of candidiasis and aspergillosis. *Eur J Clin Microbiol Infect Dis* **2013**, *32*, 387–397, doi:10.1007/s10096-012-1754-z.

29. Perlin, D. S. Echinocandin Resistance in Candida. *Clin. Infect. Dis.* **2015**, *61*, S612–S617, doi:10.1093/cid/civ791.

30. Chapman, B.; Slavin, M.; Marriott, D.; Halliday, C.; Kidd, S. E.; Arthur, I.; Bak, N.; Heath, C. H.; Kennedy, K.; Morrissey, C. O.; Sorrell, T. C.; van Hal, S.; Keighley, C.; Goeman, E.; Underwood, N.; Hajkowicz, K.; Hofmeyr, A.; Leung, M.; Macesic, N.; Botes, J.; Blyth, C.; Cooley, L.; George, C. R.; Kalukottege, P.; Kesson, A.; McMullan, B.; Baird, R.; Robson, J.; Korman, T. M.; Pendle, S.; Weeks, K.; Liu, E.; Cheong, E.; Chen, S. C. A.; Daveson, K.; Kennedy, K.; Wilson, H.; Underwood, N.; Hajkowicz, K.; Thomas, M.; Robson, J.; Peachey, G.; Cheong, E.; Gottlieb, T.; Lui, E.; Pendle, S.; Kalukottege, R. P.; Hofmeyr, A.; Jozwiak, F.; George, C. R.; Bell, S. M.; van Hal, S.; Menon, V.; Kotsiou, G.; Weeks, K.; George, C. R.; Bell, S. M.; Marriott, D.; Goeman, E.; McMullan, B.; Kesson, A.; Botes, J.; Chen, S. C. A.; Sorrell, T. C.; Keighley, C.; Halliday, C.; Baird, R.; Marshall, C.; Bak, N.; Ferguson, C.; Kidd, S. E.; Cooley, L.; Morrissey, C. O.; Korman, T. M.; Bryant, M.; Slavin, M.; Reed, C.; Slavin, M.; Darvall, J.; Maddigan, V.; Blyth, C.; Leung, M.; Arthur, I.; Heath, C. H.; Boan, P. Changing epidemiology of candidaemia in Australia. *J. Antimicrob. Chemother.* **2017**, *72*, 1103–1108, doi:10.1093/jac/dkw422.

31. Scott, L. J. Micafungin: a review of its use in the prophylaxis and treatment of invasive Candida infections. *Drugs* **2012**, *72*, 2141–65, doi:10.2165/11209970-000000000-00000.

32. Williams, D. W.; Wilson, M. J.; Lewis, M. A. O.; Potts, A. J. C. Identification of Candida Species by PCR and Restriction Fragment Length Polymorphism Analysis of Intergenic Spacer Regions of Ribosomal DNA. *J. Clin. Microbiol.* **1995**, *33*, 2476–2479.

33. Arendrup, M. C.; Arikan, S.; Barchiesi, F.; Bille, J.; Dannaoui, E.; Denning, D. W.; Donnelly, J. P.; Fegeler, W.; Moore, C.; Richardson, M.; Schmalreck, A.; Velegraki, A.; Verweij, P. EUCAST Technical Note on the method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia – forming moulds. *ESCMID Tech. Notes* **2008**, *14*, 982–984, doi:10.1111/j.1469-0691.2008.02086.x.

34. EUCAST *Breakpoint tables for interpretation of MICs, Version 8.1, valid from 2017-03-01*;

35. Rodrigues, C. F.; Gonçalves, B.; Rodrigues, M. E.; Silva, S.; Azeredo, J.; Henriques, M. The Effectiveness of Voriconazole in Therapy of Candida glabrata's Biofilms Oral Infections and Its Influence on the Matrix Composition and Gene Expression. *Mycopathologia* **2017**, *182*, 653–664, doi:10.1007/s11046-017-0135-7.

36. DuBois, M.; Gilles, K.; Hamilton, J.; Rebers, P.; Smith, F. Colorimetric Method for Determination of Sugars and Related Substances. *Anal. Chem.* **1956**, *28*, 350–356, doi:10.1021/ac60111a017.

37. Hawser, S. P.; Douglas, L. J. Resistance of Candida albicans biofilms to antifungal agents in vitro. *Antimicrob. Agents Chemother.* **1995**, *39*, 2128–2131.

38. Uppuluri, P.; Chaturvedi, A.; Srinivasan, G.; Banerjee, M.; Ramasubramaniam, A.; et al Dispersion as an important step in the Candida albicans biofilm developmental cycle. *PLoS Pathog* **2010**, 6:e1000828.

39. Taff, H.; Nett, J.; Andes, D. Comparative analysis of Candida biofilm quantitation assays. *Med Mycol* **2012**, *50*, 214–218.

40. Heffner, D.; Franklin, W. Endocarditis caused by Torulopsis glabrata. *Am J Clin Pathol* **1978**, *70*, 420–423.

41. Tumbarello, M.; Fiori, B.; Trecarichi, E. M.; Posteraro, P.; Losito, A. R.; de Luca, A.; Sanguinetti, M.; Fadda, G.; Cauda, R.; Posteraro, B.; M, T.; B, F.; EM, T.; P, P.; Al, L.; et al; Tumbarello, M.; Fiori, B.; Trecarichi, E. M.; Posteraro, P.; Losito, A. R.; de Luca, A.; Sanguinetti, M.; Fadda, G.; Cauda, R.; Posteraro, B. Risk factors and outcomes of candidemia caused by biofilm-forming isolates in a tertiary care hospital. *PLoS One* **2012**, *7*, 1–9, doi:10.1371/journal.pone.0033705.

42. Rodrigues, C. F.; Silva, S.; Azeredo, J.; Henriques, M. Candida glabrata's recurrent infections: biofilm formation during Amphotericin B treatment. *Lett. Appl. Microbiol.* **2016**, *63*, 77–81, doi:10.1111/lam.12600.

43. Cataldi, V.; Di Campli, E.; Fazii, P.; Traini, T.; Cellini, L.; Di Giulio, M. *Candida* species isolated from different body sites and their antifungal susceptibility pattern: Cross-analysis of *Candida albicans* and *Candida glabrata* biofilms. *Med. Mycol.* **2016**, myw126, doi:10.1093/mmy/myw126.

44. Rodrigues, C. F.; Rodrigues, M. E.; Silva, S.; Henriques, M. Candida glabrata Biofilms: How Far Have We Come? *J. Fungi* **2017**, *3*, 11, doi:10.3390/JOF3010011.

45. Pfaller, M. A.; Castanheira, M. Nosocomial candidiasis: Antifungal stewardship and the importance of rapid

diagnosis. *Med. Mycol.* 2016, *54*.
46. Gil-Alonso, S.; Jauregizar, N.; Cantón, E.; Eraso, E.; Quindós, G. *In Vitro* Fungicidal Activities of Anidulafungin, Caspofungin, and Micafungin against Candida glabrata, Candida bracarensis, and Candida nivariensis Evaluated by Time-Kill Studies. *Antimicrob. Agents Chemother.* **2015**, *59*, 3615–3618, doi:10.1128/AAC.04474-14.
47. Gil-Alonso, S.; Jauregizar, N.; Ortega, I.; Eraso, E.; Suárez, E.; Quindós, G. In vitro pharmacodynamic modelling of anidulafungin against Candida spp. *Int. J. Antimicrob. Agents* **2016**, *47*, 178–183, doi:10.1016/j.ijantimicag.2015.12.011.
48. Perlin, D. S. Echinocandin Resistance, Susceptibility Testing and Prophylaxis: Implications for Patient Management. *Drugs* **2014**, *74*, 1573–1585, doi:10.1007/s40265-014-0286-5.
49. Taylor, P. R.; Tsoni, S. V.; Willment, J. A.; Dennehy, K. M.; Rosas, M.; Findon, H.; Haynes, K.; Steele, C.; Botto, M.; Gordon, S.; Brown, G. D. Dectin-1 is required for ?-glucan recognition and control of fungal infection. *Nat. Immunol.* **2007**, *8*, 31–38, doi:10.1038/ni1408.
50. Gow, N. A. R.; Netea, M. G.; Munro, C. A.; Ferwerda, G.; Bates, S.; Mora-Montes, H. M.; Walker, L.; Jansen, T.; Jacobs, L.; Tsoni, V.; Brown, G. D.; Odds, F. C.; Van der Meer, J. W. M.; Brown, A. J. P.; Kullberg, B. J. Immune recognition of Candida albicans beta-glucan by dectin-1. *J. Infect. Dis.* **2007**, *196*, 1565–71, doi:10.1086/523110.
51. Scorzoni, L.; de Paula e Silva, A. C. A.; Marcos, C. M.; Assato, P. A.; de Melo, W. C. M. A.; de Oliveira, H. C.; Costa-Orlandi, C. B.; Mendes-Giannini, M. J. S.; Fusco-Almeida, A. M. Antifungal Therapy: New Advances in the Understanding and Treatment of Mycosis. *Front. Microbiol.* **2017**, *8*, 1–23, doi:10.3389/fmicb.2017.00036.
52. Ferrari, S.; Sanguinetti, M.; De Bernardis, F.; Torelli, R.; Posteraro, B.; Vandeputte, P.; Sanglard, D. Loss of mitochondrial functions associated with azole resistance in Candida glabrata results in enhanced virulence in mice. *Antimicrob. Agents Chemother.* **2011**, *55*, 1852–60, doi:10.1128/AAC.01271-10.
53. Al-fattani, M. a; Douglas, L. J. Penetration of Candida Biofilms by Antifungal Agents. *Antimicrob Agents Chemother* **2004**, *48*, 3291–3297, doi:10.1128/AAC.48.9.3291.
54. De Luca, C.; Guglielminetti, M.; Ferrario, A.; Calabrò, M.; Casari, E. Candidemia: species involved, virulence factors and antimycotic susceptibility. *New Microbiol.* **2012**, *35*, 459–468.
55. Grandesso, S.; Sapino, B.; Mazzuccato, S.; Solinas, M.; Bedin, M.; D'Angelo, M.; Gion, M. Study on in vitro susceptibility of Candida spp. isolated from blood culture. *Infect Med.* **2012**, *20*, 25–30.
56. Lewis, R.; Kontoyiannis, D.; Darouiche, R.; Raad, I.; Prince, R. Antifungal activity of amphotericin B, fluconazole, and voriconazole in an in vitro model of Candida catheter-related bloodstream infection. *Antimicrob Agents Chemother.* **2002**, *46*, 3499–505.
57. Naicker, S. D.; Magobo, R. E.; Zulu, T. G.; Maphanga, T. G.; Luthuli, N.; Lowman, W.; Govender, N. P. Two echinocandin-resistant Candida glabrata FKS mutants from South Africa. *Med. Mycol. Case Rep.* **2016**, *11*, 24–26, doi:10.1016/j.mmcr.2016.03.004.
58. Arendrup, M. C.; Perlin, D. S. Echinocandin resistance: an emerging clinical problem? *Curr. Opin. Infect. Dis.* **2014**, *27*, doi:10.1097/QCO.0000000000000111.
59. Arendrup, M. C.; Pfaller, M. a Caspofungin Etest susceptibility testing of Candida species: risk of misclassification of susceptible isolates of C. glabrata and C. krusei when adopting the revised CLSI caspofungin breakpoints. *Antimicrob. Agents Chemother.* **2012**, *56*, 3965–8, doi:10.1128/AAC.00355-12.
60. Arendrup, M.; Perlin, D.; Jensen, R.; Howard, S.; Goodwin, J.; Hopec, W. Differential in vivo activities of anidulafungin, caspofungin, and micafungin against Candida glabrata isolates with and without FSK resistance mutations. *Antim Agents Chemoter.* **2012**, 2435–2442.
61. Pham, C. D.; Iqbal, N.; Bolden, C. B.; Kuykendall, R. J.; Harrison, L. H.; Farley, M. M.; Schaffner, W.; Beldavs, Z. G.; Chiller, T. M.; Park, B. J.; Cleveland, A. a.; Lockhart, S. R. Role of FKS mutations in Candida glabrata: MIC values, echinocandin resistance, and multidrug resistance. *Antimicrob. Agents Chemother.* **2014**, *58*, 4690–4696, doi:10.1128/AAC.03255-14.
62. Pfaller, M. A.; Boyken, L.; Hollis, R. J.; Kroeger, J.; Messer, S. A.; Tendolkar, S.; Jones, R. N.; Turnidge, J.; Diekema, D. J. Wild-Type MIC Distributions and Epidemiological Cutoff Values for the Echinocandins and *Candida* spp. *J. Clin. Microbiol.* **2010**, *48*, 52–56, doi:10.1128/JCM.01590-09.
63. Tortorano, A. M.; Prigitano, A.; Lazzarini, C.; Passera, M.; Deiana, M. L.; Cavinato, S.; De Luca, C.; Grancini, A.; Lo Cascio, G.; Ossi, C.; Sala, E.; Montagna, M. T. A 1-year prospective survey of candidemia in Italy and changing epidemiology over one decade. *Infection* **2013**, *41*, 655–662, doi:10.1007/s15010-013-0455-6.
64. Pfaller, M. A.; Boyken, L.; Hollis, R. J.; Kroeger, J.; Messer, S. A.; Tendolkar, S.; Diekema, D. J. In Vitro Susceptibility of Invasive Isolates of Candida spp. to Anidulafungin, Caspofungin, and Micafungin: Six Years of Global Surveillance. *J. Clin. Microbiol.* **2008**, *46*, 150–156, doi:10.1128/JCM.01901-07.
65. Williams, C.; Ramage, G. Fungal Biofilms in Human Disease. In *Advances in Experimental Medicine and Biology*, 2015; Vol. 831, pp. 11–27 ISBN 978-3-319-09781-7.
66. Falcone, M.; Barzaghi, N.; Carosi, G.; Grossi, P.; Minoli, L.; Ravasio, V.; Rizzi, M.; Suter, F.; Utili, R.; Viscoli, C.; Venditti, M.; Italian Study on Endocarditis Candida Infective Endocarditis. *Medicine (Baltimore).* **2009**, *88*, 160–168,

doi:10.1097/MD.0b013e3181a693f8.
67. Dutronc, H.; Dauchy, F. A.; Cazanave, C.; Rougie, C.; Lafarie-Castet, S.; Couprie, B.; Fabre, T.; Dupon, M. Candida prosthetic infections: Case series and literature review. *Scand. J. Infect. Dis.* **2010**, *42*, 890–895, doi:10.3109/00365548.2010.498023.
68. Cateau, E.; Rodier, M.-H.; Imbert, C. In vitro efficacies of caspofungin or micafungin catheter lock solutions on Candida albicans biofilm growth. *J. Antimicrob. Chemother.* **2008**, *62*, 153–155, doi:10.1093/jac/dkn160.
69. Cateau, E.; Berjeaud, J.-M.; Imbert, C. Possible role of azole and echinocandin lock solutions in the control of Candida biofilms associated with silicone. *Int. J. Antimicrob. Agents* **2011**, *37*, 380–384, doi:10.1016/j.ijantimicag.2010.12.016.
70. Perlin, D. S.; Shor, E.; Zhao, Y. Update on Antifungal Drug Resistance. *Curr. Clin. Microbiol. Reports* **2015**, *2*, 84–95, doi:10.1007/s40588-015-0015-1.
71. Farmakiotis, D.; Tarrand, J. J. J.; Kontoyiannis, D. P. D. P. Drug-Resistance Candida glabrata infection in Cancer Patients. *Emerg. Infect. Dis.* **2014**, *20*, 11–26, doi:10.3201/eid2011.140685.
72. Jabra-rizk, M. A.; Falker, W. A.; Meiller, T. F. Fungal Biofilms and Drug Resistance. *Emerg. Infect* **2004**, *10*, 19, doi:10.1007/5584.
73. Wiederhold, N. P. Paradoxical echinocandin activity: a limited in vitro phenomenon? *Med. Mycol.* **2009**, *47 Suppl 1*, S369-75, doi:10.1080/13693780802428542.
74. Chandra, J.; Kuhn, D.; Mukherjee, P.; Hoyer, L.; McCormick, T.; Ghannoum, M. Biofilm formation by the fungal pathogen Candida albicans: development, architecture, and drug resistance. *J Bacteriol* **2001**, *183*, 5385–5394.
75. DM, K.; J, C.; PK, M.; MA, G. Comparison of biofilms formed by Candida albicans and Candida parapsilosis on bioprosthetic surfaces. *Infect Immun.* **2002**, *70*, 878–888.

VI

Chapter VI

Candida glabrata biofilms matrix genes' regulation

The work presented in this chapter was adapted from:

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VI.1
Portrait of matrix genes expression in *C. glabrata* biofilms with stress induced by different drugs

ABSTRACT

Background: Candida glabrata is one of the most significant *Candida* spp. associated to severe cases of candidiasis. Biofilm formation is an important feature, closely associated with antifungal resistance, involving alteration of genes' expression or mutations, which can result in treatment failure. So, the main goal of this work was to evaluate the role of a set of genes, associated to matrix production, on the *C. glabrata* biofilm resistance to antifungal drugs. Methods: the determination of the expression of *BGL2, XOG1, FKS1, FKS2, GAS2, KNH1, UGP1* and *MNN2*, in 48-h biofilm's cells of three *C. glabrata* strains, was performed through RT-qPCR, after contact with Fluconazole (Flu), Amphotericin B (AmB), Caspofungin (Csf) or Micafungin (Mcf).

Results: Mcf showed to induce a general overexpression of the selected genes. It was verified that the genes related with the production of β-1,3-glucans (*BGL2, XOG1, GAS2*) demonstrated to have the highest expressions.

Conclusion: though β-1,6-glucans and mannans are an essential part of the cell and biofilm matrix, *C. glabrata* biofilm cells seem to contribute more for the replacement of β-1,3-glucans. Thus, these biopolymers seem to have a greater impact on the biofilm matrix composition and, consequently, a role in the biofilm resistance to the antifungal drugs.

Keyword: *Candida*, biofilms, matrix, drug resistance, gene expression, *Candida glabrata.*

VI.1.1. Introduction

Fungal infections continue to grow in the last decades, particularly among immunosuppressed patients, individuals under prolonged hospitalization, catheterization or continued antimicrobial treatments [1–3]. *Candida* spp. are the commonest fungal species involved in these diseases. *Candida albicans* is the most isolated species, but *Candida glabrata* and *Candida parapsilosis* are the second most isolated in the United States of America and Europe, respectively [1,4,5]. Though *C. glabrata* does not have capacity to form hyphae, pseudohyphae or to secret proteases, they possess many other virulence factors, such as the ability to secrete phospholipases, lipases and haemolysins and, importantly, the capacity to form biofilms [6–8]. Those factors highly contribute for an extreme aggressiveness, resulting in a low therapeutic response and severe cases of recurrent candidiasis[8,9]. Biofilms are communities of microorganisms that colonize tissues and indwelling medical devices, embedded in an extracellular matrix [10,11]. These heterogeneous structures provide high resistance to antifungal therapy and strong host immune responses [7,8,12]. *Candida glabrata* has shown to form a compact biofilm structure in different multilayers [6,7], with proteins, carbohydrates and ergosterol into their matrices [6,7,13].

Numerous reports have shown the presence of β-1,3-glucans in the biofilm matrices of *C. albicans* [14–17]. Interestingly, it has been demonstrated that an increase in cell wall glucan was associated with biofilm growth [14] and, more recently, β-1,3-glucans were shown to be also present in the matrices of *C. glabrata* biofilms [13,18,19]. This specific carbohydrate, has been associated to general increase of extracellular matrix delivery, which is critical for securing biofilm cells to a surface and crucial to develop an antifungal drug resistance phenotype [14,19–23]. Several genes are involved in the delivery and the accumulation of extracellular matrix. It is recognized that, in *C. albicans,* the major β-1,3-glucan synthase are encoded mainly by *FKS1* but also by *FKS2* [24]. The *BGL2* and *XOG1* genes also have important roles in glucan matrix delivery, by encoding glucanosyltransferases and β-1,3-exoglucanase, respectively [25,26]. These genes play an important part in cell wall remodelling, however, the influence in the matrix glucan delivery does not appear to affect the quantity of cell wall ultrastructure or β-1,3-glucan concentration, suggesting that these enzymes function specifically for matrix delivery [17,19,26–28]. Likewise, identically to *S. cerevisiae*, in *C. glabrata,* the *GAS* gene family is a regulator in production of β-1,3-glucan [29]. *Gas2*, a glycosylphosphatidylinositol (GPI)-anchored cell surface protein [30,31], is a specify putative carbohydrate-active enzyme, that may change cell wall polysaccharides [29,32].

Another carbohydrate of the *C. glabrata* cell wall is β-1,6-glucan, where it occurs as a polymer covalently attached to glycoproteins [33–36], to β-1,3-glucan and chitin [37]. Nagahashi et al [36] reported the isolation of the *KNH1* homologs (genes encoding cell surface O-glycoproteins), suggesting evolutionary conservation of these molecules as essential components of β-1,6-glucan synthesis in *C. glabrata,* which was also discussed before [35,38]. Additionally, the *UGP1* gene is a putative UDP-glucose pyrophosphorylase related to the general β-1,6-D-glucan biosynthetic process [39,40]. During stress conditions, several *S. cerevisiae* genes orthologous are induced in *C. glabrata*. In glucose starvation stress, *UGP1* is induced [39].

Candida spp. external layer cell wall also consists of highly glycosylated mannoproteins [41–43], which play a major role in host recognition, adhesion and cell wall integrity [44–56]. These proteins have both N- and O-linked sugars, predominantly mannans, which are also known to be present on the biofilm matrices of *C. albicans* [57–59]. The *MNN2* gene is one putative element of the N-linked glycosylation, directly responsible for the mannans production for both cell and biofilm matrices of *C. glabrata* [57–59].

The goal of this work was to determine the profile of expression of selected genes (Table VI.1.2) related to the production of biofilm matrix components, in response to a stress caused by drugs from the most important antifungal classes: azoles (Fluconazole, Flu), polyenes (Amphotericin B, AmB) and echinocandins (Caspofungin, Csf and Micafungin, Mcf).

VI.1.2. Materials and Methods

VI.1.2.1 Organisms

Three strains of *C. glabrata* were used in the course of this study. One reference strain from the American Type Culture Collection (*C. glabrata* ATCC 2001), one strain recovered from urinary tract (*C. glabrata* 562123) and one strain recovered from vaginal tract (*C. glabrata* 534784), from Hospital Escala Braga, Portugal. The identity of all isolates was confirmed using CHROMagar™ *Candida* (CHROMagar™, Paris, France) and by PCR-based sequencing using specific primers (*ITS1* and *ITS4*) against the 5.8s subunit gene reference [60]. The PCR products were sequenced using the ABI-PRISM Big Dye terminator cycle sequencing kit (Perkin Elmer, Applied Biosystems, Warrington, UK).

VI.1.2.2 Growth conditions

For each experiment, *C. glabrata* ATCC2001, *C. glabrata* 534784 and *C. glabrata* 562123 strains were subcultured on Sabouraud dextrose agar (SDA) (Merck, Darmstadt, Germany) during 24 h at 37°C. Cells were then inoculated in Sabouraud dextrose broth (SDB) (Merck, Darmstadt, Germany) and incubated for 18 h at 37°C under agitation at 120 rpm. After incubation, the cells were harvested by centrifugation at 3000 *g* for 10 min at 4°C and washed twice with Phosphate Buffered Saline (PBS 0.1 *M*, pH=7.5). Pellets were then suspended in RPMI-1640 (pH=7, Sigma-Aldrich, Roswell Park) and the cellular density was adjusted to $1x10^5$ cells/mL, using a Neubauer counting chamber.

VI.1.2.3 Antifungal drugs

Flu, Csf and Mcf were kindly provided by Pfizer®, S.A., MSD® and Astellas® respectively, in its pure compound. AmB was purchased at Sigma® (Sigma-Aldrich, Roswell Park). Aliquots of 5000 mg/L were prepared using dimethyl-sulfoxide (DMSO). The final concentrations used were prepared with RPMI-1640.

VI.1.2.4 Biofilm formation

The minimum biofilm eradicatory concentration (MBEC) values were determined previously by the group, according to the the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines [61,62]. For the biofilm formation, standardized cell suspensions (1000 µL) were placed into selected wells of 24-wells polystyrene microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium). At 24 h, 500 µL of RPMI-1640 was removed and an equal volume of fresh RPMI-1640 plus the antifungal concentration were added, based in the MBEC values determined and indicated in bold in Table VI1.1 (2x concentrated). The plates were incubated at 37°C for more 24 h at 120 rpm. RPMI-1640 was used without cells, but with antifungal agent, as a negative control. As positive control, cell suspensions were tested without antifungal agent [18].

Table VI.1.1. MBEC concentrations (mg/L) of the *C. glabrata* strains for fluconazole (Flu), amphotericin B (AmB), caspofungin (Csf) and micafungin (Mcf)

Origin	Strain	Flu	AmB	Csf	Mcf
Reference (Wild Type)	ATCC2001	**>1250**	**4**	2.5-**3**	16-**17**
Urinary Tract	562123	**625**	**2**	0.5-**1**	16-**17**
Vaginal Tract	534784	**>1250**	**2**	2.5-**3**	5.5-**6**

***Bold**: concentrations applied to the pre-formed-biofilms.*

VI.1.2.5 Gene expression analysis

VI.1.2.5.1 Gene selection and primer design for quantitative real-time PCR.

Genes related to the production of biofilm matrix components (β-1,3, β-1,6 glucans and mannans) - *BGL2, FKS1, FKS2, GAS2, KNH1, UGP1*, *XOG1* and *MNN2* - were selected for this study. The gene sequences of interest were obtained from *Candida* Genome Database and the primers for quantitative real-time PCR (qRT-PCR) were designed using Primer 3 (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) web-based software and are listed in Table VI.1.2. *ACT1* was chosen to be the housekeeping gene. In order to verify the specificity of each primer pair for its corresponding target gene, PCR products were first amplified from *C. glabrata* ATCC2001.

VI.1.2.5.2 Preparation of biofilm cells for RNA extraction.

After, biofilm formation the medium was aspired and the wells were washed with sterile water to remove non-adherent cells. The biofilms were scraped from wells with 1 mL of sterile water and sonicated (Ultrasonic Processor, Cole-Parmer, Illinois, USA) for 30 s at 30 W to separate the cells from the biofilm matrix. Cells were harvested by centrifugation at 8000 *g* for 5 min at 4ºC[18].

VI.1.2.5.3 RNA extraction.

RNA extraction was performed using PureLink RNA Mini Kit (Invitrogen, Carlsbad, USA). Prior to RNA extraction, a lysis buffer (PureLink RNA Mini kit) was prepared adding 1% of ß-mercaptoethanol. Then, 500 µL of lysis buffer and glass beads (0.5 mm diameter) were added to each pellet. These mixes were homogenized twice for 30 s using a Mini-Bead-Beater-8 (Stratech Scientific, Soham, UK). After cell disruption, the PureLink RNA Mini Kit (Invitrogen, Carlsbad, USA) was used for total RNA extraction according to the manufacturer's recommended protocol. To avoid potential DNA contamination samples were treated with RNase-Free DNase I (Invitrogen, Carlsbad, USA) [18].

VI.1.2.5.4 Synthesis of cDNA.

To synthesize the complementary DNA (cDNA) the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, USA) was used according to the manufacturer's instructions. For each sample 10 µL of the extracted RNA was used at a final reaction volume of 50 µL. cDNA synthesis was performed firstly

at 70ºC for 5 min and then at 42ºC for 1 h. The reaction was stopped by heating for 5 min at 95ºC [18].

Table V I.1.2. Primers, targets used and specific function the genes used for the expression analysis

Sequence (5'→ 3')	Primer	Target	Properties and proposed function[a]
5'-GGC AAG AAA CTG GAC AGA GC-3'	F	*BGL2*	*β-1,3 glucanosyltransferase activity; glucan endo-β-1,3-D-glucosidase activity*
5'-GGA AAA CTT GGG TCC TGC TG-3'	R		
5'-GTC CTA ACC TTG CAC ACC AG-3'	F	*FKS1*	*β-1,3-D-glucan synthase activity*
5'-CTA CGC CCA AAC ATC AGC-3'	R		
5'-GGG TCA CTG TGA AAT GTT-3	F	*FKS2*	*β-1,3-D-glucan synthase activity*
5'-GTA GAC GGG TTC GGA TT-3	R		
5'-ACC AGT CGT ACC ATT ACC GG-3'	F	*GAS2*	*β-1,3-glucanosyltransferase activity*
5'-CCT GCC CAA CTT CTA ACA GC-3'	R		
5'-CGG TGC CAA CGG TTA CTA-3'	F	*KNH1*	*β-1,6-D-glucan biosynthetic process*
5'-GTG ACA CGG GTT TCA GGA-3'	R		
5'-AAT CGC ACA AGG CAG AGA-3'	F	*UGP1*	*β-1,6-D-glucan biosynthetic process*
5'-ACT TGG GCG ACT TCC AAT-3'	R		
5'-GGT GAG TTG CAA CGT GAC AT-3'	F	*XOG1*	*Glucan endo-β-1,6 and 1,3 glucosidase activities*
5'-ATT CGG TTA AAG CGG CAC TC-3'	R		
5'-GAA GCC TGA TGG TGG TGA-3'	F	*MNN2*	*α-mannosyltransferase biosynthetic process*
5'-ATT GGG CGA TGA CCT TCT-3'	R		
5'- GTT GAC CGA GGC TCC AAT GA-3'	F	*ACT1*	*Housekeeping gene*
5'- CAC CGT CAC CAG AGT CCA AA-3'	R		

[a]*CGD, Candida Genome Database (http://candidagenome.org/); F – forward; R – reverse.*

VI.1.2.5.5 Quantitative Real-Time PCR

Real-time PCR (qRT-PCR) (CFX96 Real-Time PCR System; Bio-Rad, Berkeley, USA) was used to determine the relative levels of all genes mRNA transcripts in the RNA samples, with *ACT1* used as a reference for *Candida* spp. housekeeping gene. Each reaction mixture consisted of a working concentration of SoFast EvaGreen Supermix (Bio-Rad, Berkeley, USA), 50 μM forward and reverse primers, and 4 μL cDNA, in a final reaction volume of 20 μL. Negative controls (water), as well as, non-transcriptase reverse controls (NRT) were included in each run [18]. The relative quantification of gene expression was performed by the $2^{-\Delta CT}$ method [63]. Each reaction was performed in triplicate and mean values of relative expression were determined for each gene. Results were presented calculating the percentage of $2^{-\Delta CT}$.

VI.1.2.6 Statistical Analysis

All experiments were repeated three times in independent assays. Results were compared using one-way ANOVA, Dunnett's post hoc multiple comparisons test, using GraphPad™ Prism 7™ (CA,

USA) software. All tests were performed with a confidence level of 95%. In order to determine the similarity of the strains genes' profile, it was also applied the Pearson Correlation Coefficient (r).

VI.3.1. Results and Discussion

Candidaemia related to *C. glabrata* has been increasing in the last years and it is related to a high drug resistance, particularly to the azole antifungal class [1,20,64]. Biofilms of *C. glabrata* are highly recalcitrant to antifungal agents' treatment, due to multiple resistance mechanisms, as the existence of a strong net of exopolysaccharydes and other biopolymers that protect the cells and difficult the drugs' diffusion [1,15,65–67]. In order to stress the *C. glabrata* biofilm cells, four antifungals were applied (based on MBECs concentration, Table VI.1.1 in pre-formed biofilms and then an evaluation of the biofilms matrix genes expression was performed.

Figure VI.1.1 shows the heatmap regarding the results of the RT-qPCR expression profiling of biofilm cells of *C. glabrata* ATCC2001 (A), *C. glabrata* 562123 (B) and *C. glabrata* 534784 (C) with antifungal contact. The final data were presented in fold-change ($2^{-\Delta CT}$) [68].

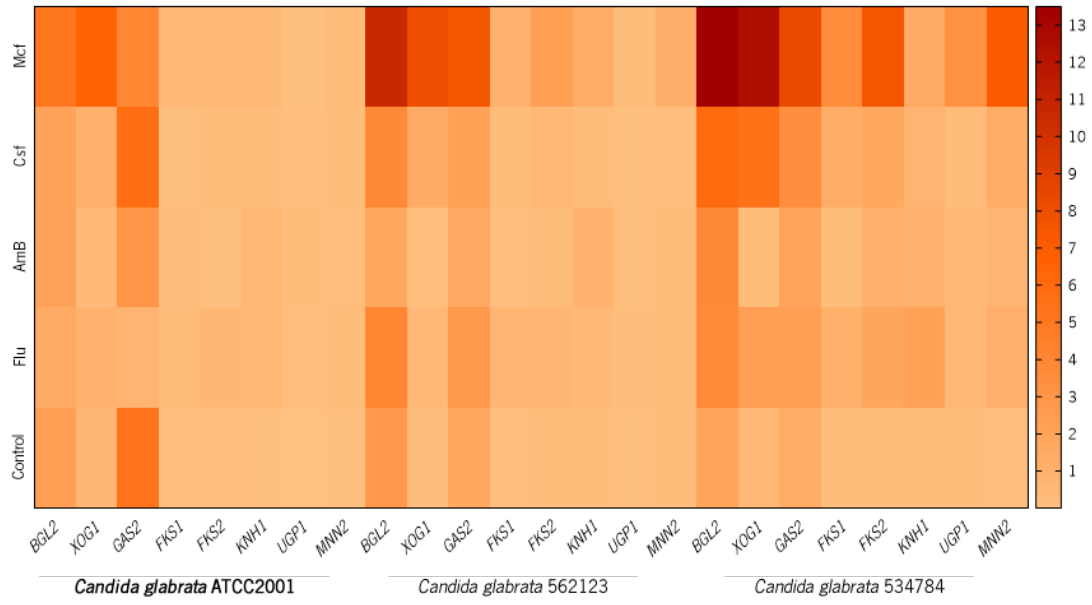

Figure VI.1.1. Real-time PCR expression profiling of *BGL2, XOG1, GAS2, FKS1, FKS2, KNH1, UGP1,* and *MNN2* genes on biofilm cells of *C. glabrata* ATCC2001, *C. glabrata* 562123 and *C. glabrata* 534784 with antifungal contact. The heatmap was generated by a log transformation of the real-time PCR and the fold change determined through $2^{-\Delta CT}$. The numerical scale in the right represents the fold change. (Flu: fluconazole; AmB: amphotericin B; Csf: caspofungin; Mcf: micafungin).

Generally, *BGL2, FKS1, FKS2, GAS2* and *XOG1* showed higher expression patterns of response and, by contrast, *KNH1*, *UPG1* and *MNN2* displayed minor expression changes in biofilm cells (Figure VI.1.1 and Table V.1.3).

BGL2 had the most similar expression in the control groups and, when in contact with the drugs, the fold change (FC) decreased for Flu and Csf, for the reference strain (FC: 1,40 and 2,00 respectively) and for AmB, for the urinary strain (FC: 1,71). In all the other cases, the fold change increased, particularly when the biofilms had contact with Mcf (FC: *C. glabrata* ATCC2001: 5,13; *C. glabrata* 562123: 10,58; *C. glabrata* 534784: 13,49). All expressions were statistically significant ($P<0.0001$). Comparing to the controls, *XOG1* revealed a statistically significant downexpression after a contact with AmB (FC: *C. glabrata* ATCC2001 0,37; *C. glabrata* 562123 0,10; *C. glabrata* 534784: 0,27), and an overexpression for all the other antifungals, for all strains. The most noteworthy overexpressions were Flu for *C. glabrata* 534784 (FC: 2,35, $P<0.0001$), Csf for *C. glabrata* 562123 and *C. glabrata* 534784 (FC: 1,42 and 5,45, $P<0.0001$, respectively) and for all strains after contact with Mcf (FC: *C. glabrata* ATCC2001: 6,54, *C. glabrata* 562123: 7,89, *C. glabrata* 534784: 12,38, all $P<0.0001$). In an important report, Taff et al[19] concluded that the *C. albicans* mutants, which were unable to produce Bgl2, Xog1 enzymes, did not appear to disturb cell wall glucan composition of biofilm cells, nor were necessary for filamentation or biofilm formation, but the biofilms had a reduced matrix glucan, reduced total matrix biomass accumulation, and improved susceptibility to antifungal drug therapy. Similarly, Li et al [69] showed that, in *C. albicans* persister cells (frequent in biofilms [70,71]), there is an increased expression of cell wall integrity proteins as Xog1 and Bgl2.The actual studies recognize a biofilm-specific pathway for Bgl2, Xog1 (and Phr1) enzymes involving the matrix delivery, in which these enzymes release and modify cell wall glucan for deposition in the extracellular space, but an alternative justification is that the enzymes act in the extracellular space, being crucial for mature matrix organization and function [19]. These enzymes have been localized on the cell wall, supporting the hypothesis of cell wall activity, but have also secretion sequences that give viability for an extracellular function. As seen earlier, *BGL2* is one of the glucan modifying genes for glucan delivery and *XOG1* is a glucanase [19], which is necessary for modification and delivery of carbohydrate to the mature biofilm matrix. Without delivery and accumulation of matrix glucan, the biofilms exhibit enhanced susceptibility to antifungal drugs [19]. This change in the regulation of *BGL2* and *XOG1* of the biofilm cells of *C. glabrata* after the drugs contact is interpreted as a response of the biofilm

cells to the reduction of the biofilm matrix, specifically β-1,3-glucans, and it has been described before [7,13,18].

Table VI.1.3. Real-time PCR expression profiling of *BGL2, FKS1, FKS2, GAS2, KNH1, UGP1*, *XOG1* and *MNN2* genes on biofilm cells of *C. glabrata* ATCC2001, *C. glabrata* 562123, *C. glabrata* 534784, with and without antifungal contact (Fold change: $2^{-\Delta CT}$). The significance of the FC results was determined comparing the treated groups with the non-treated *(* P<0.05; ** P<0.001; *** P<0.0005; **** P< 0.0001)*

Gene		*Candida glabrata* ATCC2001	*Candida glabrata* 562123	*Candida glabrata* 534784
			Fold change	
BGL2	***Non treated***	***2,23***	***2,77***	***1,90***
	Flu	1,41****	4,01****	3,66****
	AmB	2,04****	1,71****	3,85****
	Csf	2,00****	3,82****	5,98****
	Mcf	5,13****	10,58****	13,49****
XOG1	***Non treated***	***0,57***	***0,15***	***0,46***
	Flu	0,96****	0,54****	2,35****
	AmB	0,37****	0,10***	0,27****
	Csf	1,08****	1,42****	5,45****
	Mcf	6.54 ****	7.89 ****	12.38 ****
GAS2	***Non treated***	***5,34***	***1,67***	***1,39***
	Flu	0,76****	2,73****	2,26****
	AmB	3,02****	1,56****	1,95****
	Csf	5,72****	2,08****	3,39****
	Mcf	3,99****	7,43****	8,18****
FKS1	***Non treated***	***0,11***	***0,11***	***0,17***
	Flu	0,22****	0,65****	1,07****
	AmB	0,16****	0,08****	0,19 (ns)
	Csf	0,07***	0,20****	1,27****
	Mcf	0,49****	0,94****	3,55****
FKS2	***Non treated***	***0,14***	***0,20***	***0,27***
	Flu	0,61****	0,64****	1,77****
	AmB	0,06***	0,19*	1,05****
	Csf	0,28****	0,55****	1,66****
	Mcf	0,43****	2,29****	7,50****
KNH1	***Non treated***	***0,06***	***0,20***	***0,22***
	Flu	0,50****	0,41****	2,08****
	AmB	0,51****	0,87****	0,94***
	Csf	0,24****	0,21(ns)	0,72****
	Mcf	0,45****	1,33****	1,43****
UGP1	***Non treated***	***0,002***	***0,07***	***0,20***
	Flu	0,10****	0,15****	0,55****
	AmB	0,17****	0,05****	0,46****
	Csf	0,01(ns)	0,06**	0,33****
	Mcf	0,04**	0,21****	3,17****
MNN2	***Non treated***	***0,02***	***0,18***	***0,13***
	Flu	0,19****	0,34****	1,17****
	AmB	0,13****	0,24****	0,71****
	Csf	0,18****	0,13***	1,40****
	Mcf	0,31****	1,21****	7,03****

(ns – non significant)

The *GAS* gene family is also a regulator in production of β-1,3-glucan and Gas2 is a glycosylphosphatidylinositol (GPI)-anchored cell surface protein [31], involved in production of β-

1,3-glucan in *C. glabrata* [29,30]. Gas2 is a documented putative carbohydrate-active enzyme, and consequently it can alter the cell wall polysaccharides, in order to build and remodel the cell wall glycan network through growth in *C. glabrata* [29]. In *C. glabrata* ATCC2001, *GAS2* was highly expressed in the non-treated group and after Csf contact (FC: 5,72), while Flu, AmB and Mcf led to a downregulation. The clinical isolates upregulated the gene in all conditions, excepting for AmB (Figure VI.1.1, all $P<0.0001$). Hence, analysing the results of *C. glabrata* 562123 and *C. glabrata* 534784, the *GAS2* network seems to be activated, also after glycan's loss via drug aggressions, in order to replace the lack of β-1,3-glucans and re-establish the biofilm cell homeostasis. All control comparisons were statistically significant ($P<0.0001$).

The resistance to echinocandins has increased from 4.9% to 12.3% between 2001 and 2010 [72] with rapid development of *FKS* mutations in *Candida* spp., especially in *C. glabrata* [73,74]. These amino acid substitutions in *FKS1* [30,75–77] and *FKS2* [30,78] are directly related to the resistance to this class of drugs: acquired *FKS* mutations[79] are reported to confer low β-(1,3)-D-glucan synthase sensitivity and higher minimum inhibitory concentration (MIC) values, which are related with clinical failure[80]; intrinsic *FKS* mutations, also end in elevated MIC levels but in a lower level of reduced β-(1,3)-D-glucan synthase sensitivity [80–82]. Generally, in the *C. glabrata* strains, the 24-h-contact with both echinocandins upregulated *FKS1* and *FKS2* genes and, in the reference strain, it upregulated *FKS1* in the presence of Mcf and both genes with both drugs. Specifying the genes, the results showed that all strains, upregulated the expression of *FKS1*, after drug exposure (statistically significant), with the exception of Csf for *C. glabrata* ATCC2001 (FC: 0,07 $P<0.001$) and AmB for *C. glabrata* 562123 (FC: 0,08; $P<0.0001$). And, for *FKS2,* the overexpressions were observed in almost all cases and for the three strains, excluding AmB for *C. glabrata* ATCC2001 (FC: 0,28; $P<0.001$) and *C. glabrata* 562123 (FC: 0,55; $P<0.05$). *C. glabrata* 534784 revealed, once more, to have the highest capacity to overexpress both genes in response to any drug stress. These differences among the strains may be related to already described *Candida* spp. intra-strains variations [62]. Bizerra et al [83] reported the occurrence of a mutation associated with the resistance phenotype against echinocandins in *C. glabrata* isolated from a single cancer patient with candidemia exposed to antifungal prophylaxis with Mcf. Arendrup et al[84] revealed that Mcf MICs of *C. glabrata FKS* hot spot mutant isolates were less raised than those obtained for the other echinocandins, showing that the efficacy of Mcf could be differentially dependent on specific *FKS* genes mutations. These reports refer singularities on *FKS* gene and Mcf, which can also be observed in our results (Figure VI.1.1). Interestingly, up and

downregulations of *FKS1* and *FKS2* were similar in the clinical isolates and paralleled to *BGL2,* which makes sense, since this gene has shown to perform, with *XOG1* (and *PHR1*), in a complementary manner in order to distribute matrix downstream of the primary β-1,3-glucan synthase encoded by *FKS1* [19]. Previous investigations also found elevated transcript levels of *FKS1, BGL2,* and *XOG1* during *in vivo C. albicans* biofilm growth when paralleled to planktonic growth, which is consistent with our results and with a role in a biofilm-specific function, such as matrix formation [85,86].

The overexpressed values obtained for *BGL1, XOG1, FKS1, FKS2* and *GAS2* after the stress conditions induced by most antifungals, endorse the impact of β-1,3-glucans in the maintenance of the cell and biofilm matrix structure.

Sequencing studies have shown that *C. glabrata* is more closely related to *S. cerevisiae* than to *C. albicans* [87], with some genes functionally interchangeable among the two species [88,89]. An important component of the cell wall and the biofilm matrix is β-1,6-glucan, which is regulated by several genes, as *KNH1.* Preceding studies have demonstrated that the *KNH1* homologs are essential components of β-1,6-glucan synthesis in *C. glabrata* [35,36,38]. In *S. cerevisiae*, many genes involved in β-1,6-glucan synthesis were isolated through mutations (*kre* [killer resistant] mutations) that are responsible for the resistance to the K1 killer toxin, which kills sensitive yeast cells after binding to β-1,6-glucan [35,38,90]. Dijkgraaf and colleagues [35] reported that, the disruption of both *KRE9* and *KNH1* was synthetically lethal for *C. glabrata* demonstrating the importance of these genes to the maintenance of cell structure. In the present study, after a drug stress, all *C. glabrata* strains upregulated this gene (Figure VI.1.1), demonstrating a clear attempt to replace these β-1,6-glucans after losses due to the aggression of the antifungals, confirming also a certain degree of relevance of these elements in the cell wall and biofilm matrix of *C. glabrata* [35,36,38]. *C. glabrata* ATCC2001 showed to upregulate the *KNH1* gene in the presence of antifungal drugs and *C. glabrata* 562123 indicated an identical pattern by marginally increasing the gene expression in these conditions. Comparing to the other two strains and excepting in presence of Flu (FC: 2,08; $P<0.0001$), *KNH1* had different regulations in the vaginal tract strain (Figure VI.1.1). *C. glabrata* 534784 demonstrated to have the highest upregulation capacity, presenting high percentages of overexpression almost for all genes (Figure VI.1.1).

During glucose starvation, a set of genes orthologous to *S. cerevisiae* is induced in *C. glabrata*, including *UGP1*, related to the β-1,6-D-glucan biosynthetic process [39,40], which shows that the environmental stress response is conserved between *S. cerevisiae* and *C. glabrata* [39]. *UPG1*

showed to have the lowest expression, comparing with other genes and controls. Nonetheless excepting for one condition and strain, after the presence of antifungal drug, several overexpression states were observed (Figure VI.1.1). The reference strain displayed overexpression in all conditions and the highest occurred in the presence of AmB (FC: 0.17; $P<0.0001$) and the lowest for Csf (FC:0,01; non-significant); the urinary tract strain revealed to have also low results for the Csf and AmB condition and the highest expression detected belonged to Flu (0,15; $P<0.0001$). Generally, *C. glabrata* 534784 demonstrated to have highest percentages for all conditions. The lowest were related to biofilm cells stressed by AmB (FC: 0,46; $P<0.0001$). Srikantha and colleagues[91] identified a set of genes that are upregulated by the transcription factor Bcr1, involved in impermeability, impenetrability, and drug resistance of *C. albicans'* biofilms. The authors concluded that the induction of overexpression of Bcr1 in weak biofilms of *C. albicans*, conferred those three characteristics and, in these cases, *UGP1* gene was downregulated [92]. This result supports the percentages in expression we obtained: since *C. glabrata* biofilms got weakened by the drugs contact, the *UGP1* expression was increased in order to balance this defect (as seen with *KNH1*). The overexpression values we obtained for both *KNH1* and *UPG1* points to the relevance of β-1,6-glucans in the maintenance of a good cell and matrix structure.

Regarding the mannans regulation, all strains showed to have a low or moderate expression of *MNN2* in the controls, but high changes arose in the presence of all drugs and particularly when Mcf was added (Figure VI.1.1). *C. glabrata* ATCC2001 demonstrated to have the lowest expression in the control group, among all strains (Figure VI.1.1). The urinary strain presented the lowest gene expressions, when compared to the no treated group (Figure VI.1.1). Flu and Mcf indicated highest *MNN2* values (FC: 0.34 and 1,21, respectively, both $P<0.0001$), while AmB and Csf revealed the lowest expressions (FC: 0,24, $P<0.0001$ and 0,13, $P<0.0005$). For *C. glabrata* ATCC2001 and *C. glabrata* 534784, the lowest regulations were associated to the biofilm cells that were stressed by AmB (FC: 0,13 and 0,71 respectively, both $P<0.0001$). When Mcf was applied, the biofilm cells of the vaginal strain showed a strong response to the stress, comparing to the other two (FC: 7,03; $P<0.0001$). Our team has even showed that *C. glabrata* ATCC2001 increases the quantities of mannans on its cell walls in the presence of those drugs (data not shown), revealing a possible adaptation of the cells to the stress caused by the antifungal drugs. Other studies reported analogous adjustments of the cell walls after environmental drug stress, which has been related with high antifungal resistance events [1,2,19,93–95], supporting these results.

Interestingly, and when compared to the rest of the genes, the present results demonstrate that, *KNH1*, *UGP1* and *MNN2* were the genes that had the less values in percentages of expression. This seems to indicate that, although β-1,6-glucans are an important part of the cell and the biofilm matrix, the cells appear to invest more in replacing the lost β-1,3-glucans, leading to consider that these have a greater significance to the maintenance of the homeostasis of the biofilm and the biofilm cells. In fact, in studies developed in our group [13,18], the total polysaccharides and β-1,3-glucans concentrations increased significantly in *C. glabrata* biofilm matrices after Flu, AmB and Mcf contact. These higher concentrations in β-1,3-glucans content, might explain part of the main biofilm resistance to the drugs and that have been formerly stated [7,96–98].

Finally, the majority of downregulations that occurred for most genes and strains, happened in the presence of AmB and, in opposition, Mcf induced the main overexpression alterations (Figure VI.1.1). AmB is a fungicidal drug and the most important antifungal polyene, used for the treatment of systemic fungal infections [99,100]. This drug works by binding to the ergosterol of the cell wall, but also inducing oxidative stress. This explains the existence of a still low reported rate of resistance and the good effectiveness of AmB [1,13,101–103]. Also, it may be associated to the lower expression profile of the genes, detected after an AmB exposure in *C. glabrata* (Figure VI.1.1). In opposition, the most acute upregulations occurred with echinocandins and particularly when Mcf was applied. This class of antifungals act by inhibiting the β-1,3-glucan synthesis [1,98,104] which affects the cell wall and the matrix composition. By overexpressing the genes related to β-1,3-glucan synthesis (*BGL2, FKS1, FKS2, GAS2, XOG1*), the cells were attempting to compensate and replace these matrices losses in β-1,3-glucan, induced by the drugs and, thus, protect and decrease the susceptibility to the antifungals VI.1 [19]. This general increase in total carbohydrates and specifically in β-1,3-glucans in *Candida* spp. biofilm matrices has been described in data from the group [7,13,18].

Regarding to the correlation between the genes' profile in *C. glabrata*, the results on the determination of the Pearson Correlation Coefficient, are displayed in Table VI.1.3.

The results showed a strong positive correlation (r near 1) between the response profile of *BGL2, XOG1, FKS1* and *MNN2* gene expression among the three strains, which means that, up and downregulation have a high tendency to occur in the same conditions for all strains. The results on the Pearson Correlation Coefficient for the profile of *FKS2* gene, revealed that the reference strain and the isolates had a moderate positive correlation This indicates that the correlation is positive, but the relationship between the gene expression is weak and the profile of the gene response is

variable. On the other side, the clinical isolates showed strong positive correlation between the profile of this gene expression.

Table VI.1.3. Pearson Correlation Coefficient (r) determined for the expression profile of *BGL2, FKS1, FKS2, GAS2, KNH1, UGP1*, *XOG1* and *MNN2* genes on biofilm cells of *C. glabrata* ATCC2001, *C. glabrata* 562123, *C. glabrata* 534784, with and without antifungal contact

Gene	ATCC2001 vs 562123	ATCC2001 vs 534784	562123 vs 534784
BGL2	0.9100	0.9145	0.9460
XOG1	0.9965	0.9459	0.9646
FKS1	0.8947	0.8723	0.8778
FKS2	0.5074	0.4481	0.9937
GAS2	-0.0514	0.1091	0.9663
KNH1	0.6427	0.8107	0.3697
UGP1	-0.1091	-0.1122	0.8519
MNN2	0.7924	0.8618	0.9728

C. glabrata ATCC2001 and the clinical isolates demonstrated a moderate positive correlation between the profile of the *GAS2* gene expression in the three strains and a weak relationship between the gene expression. The clinical isolates had strong positive correlation between the profile of the *GAS2* gene expression in the strains. The *KNH1* gene showed to be the most variable and the less possible to correlate between strains. The reference and 562123 have a moderate correlation; reference and 534784 have a strong correlation, and the clinical isolates show here the only weak correlation detected. In what concerns the *UPG1* gene, and, although there is a negative correlation, the relationship between the gene expression in ATCC2001 and the clinical isolates could be considered as weak. Between the isolates, it was determined that the up and downregulation has a high tendency to occur in the same conditions for all strains (thus, strong correlation). In summary, *BGL2, XOG1, FKS1* and *MNN2* showed to be the genes that induced the most similar responses within the transcriptome of the three strains and the clinical isolates seen to be nearer each other than the reference strain and the clinical isolates. Once more, the β-1,3-glucan synthesis is exposed as important among *C. glabrata* (three of the four genes are responsible for their production). These similarities among these two strains may be because both are derived from hospital environment and it is probable that have already been challenged by several drugs, having their responses prompter, on contrary to the reference that is a wild type strain.

VI.4.1. Conclusions

The *in vitro* high-dose paradox associated to *Candida* spp. isolates is being increasingly reported and connected to slightly elevated MICs, potentially contributing to clinical resistance and treatment failure. These drug tolerances and associated adaptive mechanisms are highly related to the *Candida* spp. biofilm forms. The *C. glabrata* extracellular matrix is crucial for mature biofilm formation, contributing not only to the adhesive nature of biofilm cells, but protecting the cells from antifungal agents and from the host immune system. Understanding the production of the biofilm matrix components and the delivery processes associated is important to the development of effective biofilm therapies. All stakeholders in this process represent potentially attractive targets for detection of, and for therapeutic interventions against candidiasis.

References

1. Rodrigues, C. F.; Rodrigues, M. E.; Silva, S.; Henriques, M. Candida glabrata Biofilms: How Far Have We Come? *J. Fungi* **2017**, *3*, 11.
2. Silva, S.; Rodrigues, C.F.; Araújo, D.; Rodrigues, M.E.; Henriques, M. Candida Species Biofilms' Antifungal Resistance. *J. Fungi* **2017**, *3*, 8.
3. Costa-Orlandi, C.; Sardi, J.; Pitangui, N.; de Oliveira, H.; Scorzoni, L.; Galeane, M.; Medina-Alarcón, K.; Melo, W.; Marcelino, M.; Braz, J.; Fusco-Almeida, A.; Mendes-Giannini, M. Fungal Biofilms and Polymicrobial Diseases. *J. Fungi* **2017**, *3*, 22.
4. McCall, A.; Edgerton, M. Real-Time Approach to Flow Cell Imaging of Candida albicans Biofilm Development. *J. Fungi* **2017**, *3*, 13.
5. CJ, N.; AD, J.; Nobile CJ, J. A.; Nobile, C. J.; Johnson, A. D. Candida albicans biofilms and human disease. *Annu Rev Microbiol* **2015**, *69*, 71–92.
6. Silva, S.; Henriques; Martins, A.; Oliveira, R.; Williams, D.; Azeredo, J. Biofilms of non-Candida albicans Candida species: quantification, structure and matrix composition. *Med Mycol.* **2009**, *47*, 681–689.
7. Fonseca, E.; Silva, S.; Rodrigues, C. F.; Alves, C. T.; Azeredo, J.; Henriques, M. Effects of fluconazole on Candida glabrata biofilms and its relationship with ABC transporter gene expression. *Biofouling* **2014**, *30*, 447–57.
8. Rodrigues, C. F.; Silva, S.; Henriques, M. Candida glabrata: A review of its features and resistance. *Eur. J. Clin. Microbiol. Infect. Dis.* **2014**, *33*, 673–688.
9. Negri, M.; Silva, S.; Henriques, M.; Oliveira, R. Insights into Candida tropicalis nosocomial infections and virulence factors. *Eur J Clin Microbiol Infect Dis* **2012**, *31*, 1399–1412.
10. Costerton, J. W.; Lewandowski, Z.; Caldwell, D. E.; Korber, D. R.; Lappin-Scott, H. M. Microbial Biofilms. *Annu. Rev. Microbiol.* **1995**, *49*, 711–745.
11. Donlan, R.; Costerton, J. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.* **2002**, *15*, 167–193.
12. Silva, S.; Negri, M.; Henriques, M.; Oliveira, R.; Williams, D. W.; Azeredo, J. Candida glabrata, Candida parapsilosis and Candida tropicalis: biology, epidemiology, pathogenicity and antifungal resistance. *FEMS Microbiol. Rev.* **2012**, *36*, 288–305.
13. Rodrigues, C. F.; Silva, S.; Azeredo, J.; Henriques, M. Candida glabrata's recurrent infections: biofilm formation during Amphotericin B treatment. *Lett. Appl. Microbiol.* **2016**, *63*, 77–81.
14. Nett, J.; Lincoln, L.; Marchillo, K.; Massey, R.; Holoyda, K.; Hoff, B.; VanHandel, M.; Andes, D. Putative role of beta-1,3 glucans in Candida albicans biofilm resistance. *Antimicrob. Agents Chemother.* **2007**, *51*, 510–20.
15. Nett, J. E.; Sanchez, H.; Cain, M. T.; Andes, D. R. Genetic Basis of *Candida* Biofilm Resistance Due to Drug-Sequestering Matrix Glucan. *J. Infect. Dis.* **2010**, *202*, 171–175.
16. Nett, J. E.; Crawford, K.; Marchillo, K.; Andes, D. R.; Nett, J.E., Crawford, K., Marchillo, K., and Andes, D. R. Role of Fks1p and matrix glucan in Candida albicans biofilm resistance to an echinocandin, pyrimidine, and polyene. *Antimicrob. Agents Chemother.* **2010**, *54*, 3505–3508.
17. Nett, J. E.; Sanchez, H.; Cain, M. T.; Ross, K. M.; Andes, D. R. Interface of Candida albicans Biofilm Matrix-Associated Drug Resistance and Cell Wall Integrity Regulation. *Eukaryot. Cell* **2011**, *10*, 1660–1669.
18. Rodrigues, C. F.; Gonçalves, B.; Rodrigues, M. E.; Silva, S.; Azeredo, J.; Henriques, M. The Effectiveness of

Voriconazole in Therapy of Candida glabrata's Biofilms Oral Infections and Its Influence on the Matrix Composition and Gene Expression. *Mycopathologia* **2017**, *182*, 653–664.
19. Taff, H. T.; Nett, J. E.; Zarnowski, R.; Ross, K. M.; Sanchez, H.; Cain, M. T.; Hamaker, J.; Mitchell, A. P.; Andes, D. R. A Candida biofilm-induced pathway for matrix glucan delivery: implications for drug resistance. *PLoS Pathog.* **2012**, *8*, e1002848.
20. Rodrigues, C.F.; Henriques, M. Oral mucositis caused by Candida glabrata biofilms: failure of the concomitant use of fluconazole and ascorbic acid. *Ther. Adv. Infect. Dis.* **2017**, *1*.
21. Lopez-Ribot, J. L. Large-Scale Biochemical Profiling of the Candida albicans Biofilm Matrix: New Compositional, Structural, and Functional Insights. *MBio* **2014**, *5*, e01333–e01314.
22. Ramage, G.; Rajendran, R.; Sherry, L.; Williams, C. Fungal biofilm resistance. *Int. J. Microbiol.* **2012**, *2012*, 528521.
23. Mitchell, K. F.; Zarnowski, R.; Andes, D. R. Fungal Super Glue: The Biofilm Matrix and Its Composition, Assembly, and Functions. *PLOS Pathog.* **2016**, *12*, e1005828.
24. Douglas, C. Fungal b (1,3)-D-glucan synthesis. *Med. Mycol.* **2001**, *39*, 55–66.
25. del Mar Gonzalez, M.; Diez-Orejas, R.; Molero, G.; alvarez, A. M.; Pla, J.; Pla, J.; Nombela, C.; Sanchez-PeArez, M. Phenotypic characterization of a Candida albicans strain deficient in its major exoglucanase. *Microbiology* **1997**, *143*, 3023–3032.
26. Sarthy, A. V.; McGonigal, T.; Coen, M.; Frost, D. J.; Meulbroek, J. A.; Goldman, R. C. Phenotype in Candida albicans of a disruption of the BGL2 gene encoding a 1,3- -glucosyltransferase. *Microbiology* **1997**, *143*, 367–376.
27. Mouyna, I.; Fontaine, T.; Vai, M.; Monod, M.; Fonzi, W. A.; Diaquin, M.; Popolo, L.; Hartland, R. P.; Latgé, J. P. Glycosylphosphatidylinositol-anchored glucanosyltransferases play an active role in the biosynthesis of the fungal cell wall. *J. Biol. Chem.* **2000**, *275*, 14882–9.
28. Goldman, R. C.; Sullivan, P. A.; Zakula, D.; Capobianco, J. O. Kinetics of beta-1,3 glucan interaction at the donor and acceptor sites of the fungal glucosyltransferase encoded by the BGL2 gene. *Eur. J. Biochem.* **1995**, *227*, 372–8.
29. de Groot, P. W. J.; Kraneveld, E. a; Yin, Q. Y.; Dekker, H. L.; Gross, U.; Crielaard, W.; de Koster, C. G.; Bader, O.; Klis, F. M.; Weig, M. The cell wall of the human pathogen Candida glabrata: differential incorporation of novel adhesin-like wall proteins. *Eukaryot. Cell* **2008**, *7*, 1951–64.
30. Garcia-Effron, G.; Lee, S.; Park, S.; Cleary, J. D.; Perlin, D. S. Effect of Candida glabrata FKS1 and FKS2 mutations on echinocandin sensitivity and kinetics of 1,3-beta-D-glucan synthase: implication for the existing susceptibility breakpoint. *Antimicrob. Agents Chemother.* **2009**, *53*, 3690–9.
31. Vai, M.; Orlandi, I.; Cavadini, P.; Alberghina, L.; Popolo, L. Candida albicans homologue of GGP1/GAS1 gene is functional in Saccharomyces cerevisiae and contains the determinants for glycosylphosphatidylinositol attachment. *Yeast* **1996**, *12*, 361–8.
32. Miyazaki, T.; Nakayama, H.; Nagayoshi, Y.; Kakeya, H.; Kohno, S. Dissection of Ire1 functions reveals stress response mechanisms uniquely evolved in Candida glabrata. *PLoS Pathog.* **2013**, *9*, e1003160.
33. Montijn, R. C.; van Rinsum, J.; van Schagen, F. A.; Klis, F. M. Glucomannoproteins in the cell wall of Saccharomyces cerevisiae contain a novel type of carbohydrate side chain. *J. Biol. Chem.* **1994**, *269*, 19338–42.
34. Kapteyn, J. C.; Montijn, R. C.; Vink, E.; de la Cruz, J.; Llobell, A.; Douwes, J. E.; Shimoi, H.; Lipke, P. N.; Klis, F. M. Retention of Saccharomyces cerevisiae cell wall proteins through a phosphodiester-linked beta-1,3-/beta-1,6-glucan heteropolymer. *Glycobiology* **1996**, *6*, 337–45.
35. Dijkgraaf, G. J.; Brown, J. L.; Bussey, H. The KNH1 gene of Saccharomyces cerevisiae is a functional homolog of KRE9. *Yeast* **1996**, *12*, 683–92.
36. Nagahashi, S.; Lussier, M.; Bussey, H. Isolation of Candida glabrata homologs of the Saccharomyces cerevisiae KRE9 and KNH1 genes and their involvement in cell wall beta-1,6-glucan synthesis. *J. Bacteriol.* **1998**, *180*, 5020–9.
37. Cid, V. J.; Durán, A.; del Rey, F.; Snyder, M. P.; Nombela, C.; Sánchez, M. Molecular basis of cell integrity and morphogenesis in Saccharomyces cerevisiae. *Microbiol. Rev.* **1995**, *59*, 345–86.
38. Brown, J. L.; Kossaczka, Z.; Jiang, B.; Bussey, H. A mutational analysis of killer toxin resistance in Saccharomyces cerevisiae identifies new genes involved in cell wall (1-6)-beta-glucan synthesis. *Genetics* **1993**, *133*, 837–49.
39. Roetzer, A.; Gregori, C.; Jennings, A. M.; Quintin, J.; Ferrandon, D.; Butler, G.; Kuchler, K.; Ammerer, G.; Schüller, C. *Candida glabrata* environmental stress response involves *Saccharomyces cerevisiae* Msn2/4 orthologous transcription factors. *Mol. Microbiol.* **2008**, *69*, 603–620.
40. Dodgson, A. R.; Pujol, C.; Denning, D. W.; Soll, D. R.; Fox, A. J. Multilocus sequence typing of Candida glabrata reveals geographically enriched clades. *J. Clin. Microbiol.* **2003**, *41*, 5709–17.
41. Ruiz-Herrera, J.; Victoria Elorza, M.; Valent??n, E.; Sentandreu, R. Molecular organization of the cell wall of *Candida albicans* and its relation to pathogenicity. *FEMS Yeast Res.* **2006**, *6*, 14–29.
42. Latg?, J.-P. The cell wall: a carbohydrate armour for the fungal cell. *Mol. Microbiol.* **2007**, *66*, 279–290.

43. Netea, M. G.; Brown, G. D.; Kullberg, B. J.; Gow, N. A. R. An integrated model of the recognition of Candida albicans by the innate immune system. *Nat. Rev. Microbiol.* **2008**, *6*, 67–78.
44. Netea, M. G.; Mardi, L. Innate immune mechanisms for recognition and uptake of Candida species. *Trends Immunol.* **2010**, *31*, 346–353.
45. Gow, N. A. R.; Netea, M. G.; Munro, C. A.; Ferwerda, G.; Bates, S.; Mora-Montes, H. M.; Walker, L.; Jansen, T.; Jacobs, L.; Tsoni, V.; Brown, G. D.; Odds, F. C.; Van der Meer, J. W. M.; Brown, A. J. P.; Kullberg, B. J. Immune recognition of Candida albicans beta-glucan by dectin-1. *J. Infect. Dis.* **2007**, *196*, 1565–71.
46. van de Veerdonk, F. L.; Kullberg, B. J.; van der Meer, J. W.; Gow, N. A.; Netea, M. G. Host?microbe interactions: innate pattern recognition of fungal pathogens. *Curr. Opin. Microbiol.* **2008**, *11*, 305–312.
47. Reid, D. M.; Gow, N. A.; Brown, G. D. Pattern recognition: recent insights from Dectin-1. *Curr. Opin. Immunol.* **2009**, *21*, 30–37.
48. Mora-Montes, H. M.; Bates, S.; Netea, M. G.; Castillo, L.; Brand, A.; Buurman, E. T.; Diaz-Jimenez, D. F.; Jan Kullberg, B.; Brown, A. J. P.; Odds, F. C.; Gow, N. A. R. A Multifunctional Mannosyltransferase Family in Candida albicans Determines Cell Wall Mannan Structure and Host-Fungus Interactions. *J. Biol. Chem.* **2010**, *285*, 12087–12095.
49. Taylor, P. R.; Tsoni, S. V.; Willment, J. A.; Dennehy, K. M.; Rosas, M.; Findon, H.; Haynes, K.; Steele, C.; Botto, M.; Gordon, S.; Brown, G. D. Dectin-1 is required for ?-glucan recognition and control of fungal infection. *Nat. Immunol.* **2007**, *8*, 31–38.
50. Murciano, C.; Moyes, D. L.; Runglall, M.; Islam, A.; Mille, C.; Fradin, C.; Poulain, D.; Gow, N. A. R.; Naglik, J. R. Candida albicans Cell Wall Glycosylation May Be Indirectly Required for Activation of Epithelial Cell Proinflammatory Responses. *Infect. Immun.* **2011**, *79*, 4902–4911.
51. Gow, N. A.; Hube, B. Importance of the Candida albicans cell wall during commensalism and infection. *Curr. Opin. Microbiol.* **2012**, *15*, 406–412.
52. Bates, S.; Hughes, H. B.; Munro, C. A.; Thomas, W. P. H.; MacCallum, D. M.; Bertram, G.; Atrih, A.; Ferguson, M. A. J.; Brown, A. J. P.; Odds, F. C.; Gow, N. A. R. Outer Chain N-Glycans Are Required for Cell Wall Integrity and Virulence of Candida albicans. *J. Biol. Chem.* **2006**, *281*, 90–98.
53. Bates, S.; MacCallum, D. M.; Bertram, G.; Munro, C. A.; Hughes, H. B.; Buurman, E. T.; Brown, A. J. P.; Odds, F. C.; Gow, N. A. R. Candida albicans Pmr1p, a Secretory Pathway P-type Ca2+/Mn2+-ATPase, Is Required for Glycosylation and Virulence. *J. Biol. Chem.* **2005**, *280*, 23408–23415.
54. Mora-Montes, H. M.; Bates, S.; Netea, M. G.; Diaz-Jimenez, D. F.; Lopez-Romero, E.; Zinker, S.; Ponce-Noyola, P.; Kullberg, B. J.; Brown, A. J. P.; Odds, F. C.; Flores-Carreon, A.; Gow, N. A. R. Endoplasmic Reticulum -Glycosidases of Candida albicans Are Required for N Glycosylation, Cell Wall Integrity, and Normal Host-Fungus Interaction. *Eukaryot. Cell* **2007**, *6*, 2184–2193.
55. Munro, C. A.; Bates, S.; Buurman, E. T.; Hughes, H. B.; MacCallum, D. M.; Bertram, G.; Atrih, A.; Ferguson, M. A. J.; Bain, J. M.; Brand, A.; Hamilton, S.; Westwater, C.; Thomson, L. M.; Brown, A. J. P.; Odds, F. C.; Gow, N. A. R. Mnt1p and Mnt2p of Candida albicans Are Partially Redundant ?-1,2-Mannosyltransferases That Participate in O-Linked Mannosylation and Are Required for Adhesion and Virulence. *J. Biol. Chem.* **2005**, *280*, 1051–1060.
56. Saijo, S.; Ikeda, S.; Yamabe, K.; Kakuta, S.; Ishigame, H.; Akitsu, A.; Fujikado, N.; Kusaka, T.; Kubo, S.; Chung, S.; Komatsu, R.; Miura, N.; Adachi, Y.; Ohno, N.; Shibuya, K.; Yamamoto, N.; Kawakami, K.; Yamasaki, S.; Saito, T.; Akira, S.; Iwakura, Y. Dectin-2 Recognition of α-Mannans and Induction of Th17 Cell Differentiation Is Essential for Host Defense against Candida albicans. *Immunity* **2010**, *32*, 681–691.
57. Lal, P.; Sharma, D.; Pruthi, P.; Pruthi, V. Exopolysaccharide analysis of biofilm-forming Candida albicans. *J. Appl. Microbiol.* **2010**, *109*, 128–136.
58. Correia, I. A. D. F. L. Role of secreted aspartyl proteases in Candida albicans virulence , host immune response and immunoprotection in murine disseminated candidiasis, 2012.
59. Johnson, C. J.; Cabezas-Olcoz, J.; Kernien, J. F.; Wang, S. X.; Beebe, D. J.; Huttenlocher, A.; Ansari, H.; Nett, J. E. The Extracellular Matrix of Candida albicans Biofilms Impairs Formation of Neutrophil Extracellular Traps. *PLOS Pathog.* **2016**, *12*, e1005884.
60. Williams, D. W.; Wilson, M. J.; Lewis, M. A. O.; Potts, A. J. C. Identification of Candida Species by PCR and Restriction Fragment Length Polymorphism Analysis of Intergenic Spacer Regions of Ribosomal DNA. *J. Clin. Microbiol.* **1995**, *33*, 2476–2479.
61. Arendrup, M. C.; Arikan, S.; Barchiesi, F.; Bille, J.; Dannaoui, E.; Denning, D. W.; Donnelly, J. P.; Fegeler, W.; Moore, C.; Richardson, M.; Schmalreck, A.; Velegraki, A.; Verweij, P. EUCAST Technical Note on the method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia – forming moulds. *ESCMID Tech. Notes* **2008**, *14*, 982–984.
62. EUCAST *Breakpoint tables for interpretation of MICs, Version 8.1, valid from 2017-03-01*.
63. Schmittgen, T. D.; Livak, K. J. Analyzing real-time PCR data by the comparative CT method. *Nat. Protoc.* **2008**, *3*, 1101–1108.

64. Cho, E.-J.; Shin, J. H.; Kim, S. H.; Kim, H.-K.; Park, J. S.; Sung, H.; Kim, M.-N.; Im, H. J. Emergence of multiple resistance profiles involving azoles, echinocandins and amphotericin B in Candida glabrata isolates from a neutropenia patient with prolonged fungaemia. *J. Antimicrob. Chemother.* **2014**, 2–3.
65. Douglas, L. J. Candida biofilms and their role in infection. *Trends Microbiol.* **2003**, *11*, 30–6.
66. Zarnowski, R.; Westler, W. M.; Lacmbouh, G. A.; Marita, J. M.; Bothe, J. R.; Bernhardt, J.; Sahraoui, A. L. H.; Fontainei, J.; Sanchez, H.; Hatfeld, R. D.; Ntambi, J. M.; Nett, J. E.; Mitchell, A. P.; Andes, D. R. Novel entries in a fungal biofilm matrix encyclopedia. *MBio* **2014**, *5*, 1–13.
67. Mukherjee, P. K.; Chandra, J. Candida biofilm resistance. *Drug Resist. Updat.* **2004**, *7*, 301–309.
68. Livak, K. J. *User Bulletin #2 ABI P RISM 7700 Sequence Detection System SUBJECT: Relative Quantitation of Gene Expression - updated 2001*; 1997.
69. Li, P.; Seneviratne, C.; Alpi, E.; Vizcaino, J.; Jin, L. Delicate metabolic control and coordinated stress response critically determine antifungal tolerance of Candida albicans biofilm persisters. *Antimicrob. Agents Chemother.* **2015**, *59*, 6101–6112.
70. Al-Dhaheri, R. S. R. S.; Douglas, L. J. J. Absence of amphotericin B-tolerant persister cells in biofilms of some Candida species. *Antimicrob. Agents Chemother.* **2008**, *52*, 1884–1887.
71. Sun, J.; Li, Z.; Chu, H.; Guo, J.; Jiang, G.; Qi, Q. Candida albicans Amphotericin B-Tolerant Persister Formation is Closely Related to Surface Adhesion. *Mycopathologia* **2016**, *181*, 41–49.
72. Alexander, B. D.; Johnson, M. D.; Pfeiffer, C. D.; Jimenez-Ortigosa, C.; Catania, J.; Booker, R.; Castanheira, M.; Messer, S. A.; Perlin, D. S.; Pfaller, M. A. Increasing echinocandin resistance in Candida glabrata: clinical failure correlates with presence of FKS mutations and elevated minimum inhibitory concentrations. *Clin. Infect. Dis.* **2013**, *56*, 1724–1732.
73. Pinhati, H. M. S.; Casulari, L. A.; Souza, A. C. R.; Siqueira, R. A.; Damasceno, C. M. G.; Colombo, A. L. Outbreak of candidemia caused by fluconazole resistant Candida parapsilosis strains in an intensive care unit. *BMC Infect. Dis.* **2016**, *16*, 433.
74. Bizerra, F. C.; Jimenez-Ortigosa, C.; Souza, A. C. R.; Breda, G. L.; Queiroz-Telles, F.; Perlin, D. S.; Colombo, A. L. Breakthrough candidemia due to multidrug-resistant Candida glabrata during prophylaxis with a low dose of micafungin. **2014**, *58*, 2438–40.
75. Park, S.; Kelly, R.; Kahn, J. N. N.; Robles, J.; Hsu, M. J. M.-J.; Register, E.; Li, W.; Vyas, V.; Fan, H.; Abruzzo, G.; Flattery, A.; Gill, C.; Chrebet, G.; Parent, S. A. A.; Kurtz, M.; Teppler, H.; Douglas, C. M. M.; Perlin, D. S. S. Specific substitutions in the echinocandin target Fks1p account for reduced susceptibility of rare laboratory and clinical Candida sp. isolates. *Antimicrob. Agents Chemother.* **2005**, *49*, 3264–3273.
76. Desnos-Ollivier, M.; Moquet, O.; Chouaki, T.; Guerin, A. M.; Dromer, F. Development of echinocandin resistance in Clavispora lusitaniae during caspofungin treatment. *J. Clin. Microbiol.* **2011**, *49*, 2304–2306.
77. Jensen, R.H., Johansen, H.K. & Arendrup, M. C. Stepwise development of a homozygous S80P substitution in Fks1p, conferring echinocandin resistance in Candida tropicalis. *Antimicrob. Agents Chemother.* **2013**, *57*, 614–617.
78. Lewis, J. S.; Wiederhold, N. P.; Wickes, B. L.; Patterson, T. F.; Jorgensen, J. H. Rapid emergence of echinocandin resistance in Candida glabrata resulting in clinical and microbiologic failure. *Antimicrob. Agents Chemother.* **2013**, *57*, 4559–4561.
79. Shields, R. K.; Nguyen, M. H.; Press, E. G.; Kwa, A. L.; Cheng, S.; Du, C.; Clancy, C. J. The presence of an FKS mutation rather than MIC is an independent risk factor for failure of echinocandin therapy among patients with invasive candidiasis due to Candida glabrata. *Antimicrob. Agents Chemother.* **2012**, *56*, 4862–9.
80. Beyda, N. D.; Lewis, R. E.; Garey, K. W. Echinocandin resistance in Candida species: mechanisms of reduced susceptibility and therapeutic approaches. *Ann. Pharmacother.* **2012**, *46*, 1086–1096.
81. Barchiesi, F.; Spreghini, E.; Tomassetti, S.; Della Vittoria, A.; Arzeni, D.; Manso, E.; Scalise, G. Effects of caspofungin against Candida guilliermondii and Candida parapsilosis. *Antimicrob. Agents Chemother.* **2006**, *50*, 2719–27.
82. Garcia-Effron, G.; Katiyar, S. K.; Park, S.; Edlind, T. D.; Perlin, D. S. A naturally occurring proline-to-alanine amino acid change in Fks1p in Candida parapsilosis, Candida orthopsilosis, and Candida metapsilosis accounts for reduced echinocandin susceptibility. *Antimicrob. Agents Chemother.* **2008**, *52*, 2305–2312.
83. Archimedes, D.; Carolina, A.; Souza, R.; Colombo, A. L. Revisiting Species Distribution and Antifungal Susceptibility of Candida Bloodstream Isolates from Latin American Medical Centers. *J. Fungi* **2017**, *3*, 24.
84. Arendrup, M.; Perlin, D.; Jensen, R.; Howard, S.; Goodwin, J.; Hopec, W. Differential in vivo activities of anidulafungin, caspofungin, and micafungin against Candida glabrata isolates with and without FSK resistance mutations. *Antim Agents Chemoter.* **2012**, 2435–2442.
85. Fanning, S.; Mitchell, A. P. Fungal Biofilms. *PLoS Pathog.* **2012**, *8*, e1002585.
86. Nett, J.; Lepak, A.; Marchillo, K.; Andes, D. Time course global gene expression analysis of an in vivo Candida biofilm. *J Infect Dis* **2009**, *200*, 307–313.

87. Barns, S. M.; Lane, D. J.; Sogin, M. L.; Bibeau, C.; Weisburg, W. G. Evolutionary relationships among pathogenic Candida species and relatives. *J. Bacteriol.* **1991**, *173*, 2250–5.
88. Kitada, K.; Yamaguchi, E.; Arisawa, M. Cloning of the Candida glabrata TRP1 and HIS3 genes, and construction of their disruptant strains by sequential integrative transformation. *Gene* **1995**, *165*, 203–6.
89. Nakayama, H.; Ueno, K.; Uno, J.; Nagi, M.; Tanabe, K.; Aoyama, T.; Chibana, H.; Bard, M. Growth defects resulting from inhibiting ERG20 and RAM2 in Candida glabrata. *FEMS Microbiol Lett* **2011**, 27–33.
90. Boone, C.; Sommer, S. S.; Hensel, A.; Bussey, H. Yeast KRE genes provide evidence for a pathway of cell wall beta-glucan assembly. *J. Cell Biol.* **1990**, *110*, 1833–43.
91. Srikantha, T.; Daniels, K. J.; Pujol, C.; Kim, E.; Soll, D. R. Identification of genes upregulated by the transcription factor Bcr1 that are involved in impermeability, impenetrability, and drug resistance of Candida albicans a/α biofilms. *Eukaryot. Cell* **2013**, *12*, 875–88.
92. T, S.; Al., E.; Srikantha, T.; Daniels, K. J.; Pujol, C.; Kim, E.; Soll, D. R. Identification of genes upregulated by the transcription factor Bcr1 that are involved in impermeability, impenetrability, and drug resistance of Candida albicans a/alpha biofilms. *Eukaryot Cell* **2013**, *12*, 875–88.
93. Chen, K.-H.; Miyazaki, T.; Tsai, H.-F.; Bennett, J. E. The bZip transcription factor Cgap1p is involved in multidrug resistance and required for activation of multidrug transporter gene CgFLR1 in Candida glabrata. *Gene* **2007**, *386*, 63–72.
94. Ferrari, S.; Sanguinetti, M.; De Bernardis, F.; Torelli, R.; Posteraro, B.; Vandeputte, P.; Sanglard, D. Loss of mitochondrial functions associated with azole resistance in Candida glabrata results in enhanced virulence in mice. *Antimicrob. Agents Chemother.* **2011**, *55*, 1852–60.
95. Mathé, L.; Van Dijck, P. Recent insights into Candida albicans biofilm resistance mechanisms. *Curr. Genet.* **2013**, *59*, 251–264.
96. Marco, F.; Pfaller, M. A.; Messer, S. A.; Jones, R. N. Activity of MK-0991 (L-743,872), a new echinocandin, compared with those of LY303366 and four other antifungal agents tested against blood stream isolates of Candida spp. *Diagn. Microbiol. Infect. Dis.* **1998**, *32*, 33–7.
97. Kuhn, D. M.; George, T.; Chandra, J.; Mukherjee, P. K.; Ghannoum, M. A. Antifungal Susceptibility of Candida Biofilms: Unique Efficacy of Amphotericin B Lipid Formulations and Echinocandins. *Antimicrob Agents Chemother* **2002**, *46*, 1773–1780.
98. Scorzoni, L.; de Paula e Silva, A. C. A.; Marcos, C. M.; Assato, P. A.; de Melo, W. C. M. A.; de Oliveira, H. C.; Costa-Orlandi, C. B.; Mendes-Giannini, M. J. S.; Fusco-Almeida, A. M. Antifungal Therapy: New Advances in the Understanding and Treatment of Mycosis. *Front. Microbiol.* **2017**, *8*, 1–23.
99. Pierce, C. G.; Srinivasan, A.; Uppuluri, P.; Ramasubramanian, A. K.; López-Ribot, J. L. Antifungal therapy with an emphasis on biofilms. *Curr. Opin. Pharmacol.* **2013**, *13*.
100. Pappas, P. G.; Kauffman, C. A.; Andes, D. R.; Clancy, C. J.; Marr, K. A.; Ostrosky-Zeichner, L.; Reboli, A. C.; Schuster, M. G.; Vazquez, J. A.; Walsh, T. J.; Zaoutis, T. E.; Sobel, J. D. Clinical Practice Guideline for the Management of Candidiasis: 2016 Update by the Infectious Diseases Society of America. *Clin. Infect. Dis.* **2015**, *62*, e1–e50.
101. Canuto, M. M.; Rodero, F. G. Antifungal drug resistance to azoles and polyenes. *Lancet Infect. Dis.* **2002**, *2*, 550–563.
102. Rex, J. H. J.; Walsh, T. J.; Sobel, J. D. J.; Filler, S. G.; Pappas, P. G.; Dismukes, W. E.; Edwards, J. E. Practice guidelines for the treatment of candidiasis. *Clin Infect Dis* **2000**, *30*, 662–78.
103. Schmalreck, A. F.; Willinger, B.; Haase, G.; Blum, G.; Lass-Flörl, C.; Fegeler, W.; Becker, K.; Antifungal Susceptibility Testing-AFST Study Group Species and susceptibility distribution of 1062 clinical yeast isolates to azoles, echinocandins, flucytosine and amphotericin B from a multi-centre study. *Mycoses* **2012**, *55*, e124-37.
104. Perlin, D. S. Mechanisms of echinocandin antifungal drug resistance. *Ann N Y Acad Sci* **2015**, 1–11.

VI.2

The *MNN2* gene knockout modulates the antifungal resistance of biofilms of *C. glabrata*

ABSTRACT

Background: Systemic candidiasis is increasing in the last decades, especially among immunosuppressed patients. *Candida glabrata* is one of the problematic species, since it is related to a high rate of resistance to antifungal treatments, especially when in biofilm form.

Objective: The goal of this study was to evaluate and understand the effect of the knock-out (KO) of the *MNN2* gene in the resistant profile of biofilm cells of *C. glabrata*.

Methods: Three strains were used *C. glabrata* ATCC2001, the strain with the *MNN2* gene KO, *C. glabrata* Δ*mnn2* and the parent strain *C. glabrata* HT6. The structure of the biofilm was visualized by Confocal laser scanning microscopy and the susceptibility was assessed for several antifungals: fluconazole (Flu), amphotericin B (AmB), caspofungin (Csf) and micafungin (Mcf). Crystal violet was performed to evaluate the variation in the biomass, after the addition of the drugs and the Alcian Blue was used to assess the mannans concentration in the biofilm cells and in the matrices. Finally, β-1,3-glucans were also determined.

Results: CLSM images revealed no visible differences among cell walls of the three strains, but, in the crystal violet assays, the mutant showed to have a great biomass reduction, after a drug stress. The susceptibility of the biofilm cells of the mutant decreased, when compared to the reference strain. The KO resulted in a significant decrease of mannans in the mutant cell walls and matrices (20% in the mutant strain and 50-60% for the reference and parent in the controls and 10% and 20%, respectively, after antifungal contact, $P<0.0005$). The biofilm cell walls composition denounced a clear increase of quantity of β-1,3-glucans in the *C. glabrata* Δ*mnn2,* when compared with the other two.

Keywords: *Candida glabrata*, echinocandin, biofilm, matrix, antifungal drug, resistance.

VI.1.2. Introduction

Candida glabrata, growing only as blastoconidia, is not a polymorphic fungus, and has a haploid genome, in opposition to the diploid genome of *Candida albicans* and other *Candida* spp. [1]. Despite of being part of the natural human microflora, hostile conditions may predispose to various infections and, under circumstances where the host immune system becomes severely compromised (e.g. chemotherapy, traumas), *C. glabrata* can invade epithelial cells, disseminating via the bloodstream and causing systemic diseases [1–3]. In fact, fungal diseases associated with non-*Candida albicans Candida* (NCAC) spp. have been globally increasing [4–6]. The frequency rates of candidemia attributed to *C. glabrata* are about 15% of all *Candida* spp.-related systemic bloodstream infections [7,8], which is extremely relevant since, compared to other *Candida* spp. infections, the mortality rate associated with *C. glabrata* is the highest (30%) [1,9–15]. Biofilm formation is a very important virulence factor for *C. glabrata*, allowing the yeasts to adhere to biotic and abiotic surfaces, developing into highly organized communities that are extremely refractory to antifungal treatment and environmental conditions [16].

The yeast cell wall is a dynamic structural organelle crucial for protection against hostile environments, for maintaining cell shape, for assisting adherence to host surfaces, has a fundamental role for fungus-host interactions and immune recognition [2,17–21]. *Per se*, it is very likely to play an important part in mediating several interactions and hence virulence [17]. The cell wall is comprised of an internal central core of β-1,3-glucans and β-1,6-glucans linked to chitin via β-1,4-glucans, extended throughout the entire depth of the cell wall structure [21,22]. There is also an external layer of highly glycosylated mannoproteins [19–21], which play a major role in host recognition, adhesion, cell wall integrity, and comprise up to 40% of the cell wall dry weight [23–35]. These proteins are adorned with both N- and O-linked sugars, mainly mannans, and can end in the accumulation of up to 200 mannose units [17,36], attached via a phosphodiester linkage (phosphomannan) [37]. The number of mannans units and theirs molecular weights highly fluctuate between species [38], which have important consequences for host-fungus interactions [2,17]. Structural studies point out that *C. glabrata* mannan is more closely related to that of *Saccharomyces cerevisiae* than to *C. albicans* [39–41]. Even though, the core of the biosynthetic machinery appears to be relatively well conserved [17]. The N-Linked protein glycosylation occurs in two stages. Concisely, in the first, proteins transverse though the endoplasmic reticulum, resulting in the mature N-mannan core, $Man_8GlcNAc_2$ [33]; in the second, a α-1,6-backbone is attached to a Och_1 core and Mnn_9 and added with side chains consisting of α-1,2-, and α-1,3-

mannose residues, with the α-1,2-mannose extending out from the backbone [31,42]. It is recognised that the deletion of Sc*MNN2* inhibits the accumulation of α-1,2-mannose onto the mannan backbone, hence stopping the elaboration of N-mannan outer chains [43]. In *C. albicans*, this has considerable costs for growth, cell morphology and immune recognition [31]. Several mannosyltransferases involved in mannan biosynthesis in *S. cerevisiae*, have been identified, and many of these enzymes are conserved in *C. albicans* and other pathogenic fungi. Remarkably, many of these fungal mannosyltransferases are absent from human cells, and thus its study shows prospective in the development of novel antifungals and vaccines. Yet, this subject is more advanced in *S. cerevisiae* and *C. albicans*, little is known regarding the role in the pathogenesis of *C. glabrata*, especially in biofilms.

Therefore, the goal of this work was to evaluate and to understand the response to several antifungals stresses, specifically on *C. glabrata* biofilm cells, after an inactivation of the *MNN2* gene, a putative component of the N-linked glycosylation machinery on their cell wall.

VI.2.2. Material and Methods

VI.2.2.1 Organisms

Three strains of *C. glabrata* were used in the course of this study. One reference strain from the American Type Culture Collection (*C. glabrata* ATCC 2001) and two strains kindly provided by Ken Haynes´ group from the University of Exeter (United Kingdom) [17]: the *C. glabrata* mutant with a *MNN2* gene disruption (*C. glabrata* Δ*mnn2*) and its parent strain (*C. glabrata* HT6). The identity of all isolates was confirmed using CHROMagar™ *Candida* (CHROMagar™, Paris, France) and by PCR-based sequencing [44]. The PCR products were sequenced using the ABI-PRISM Big Dye terminator cycle sequencing kit (Perkin Elmer, Applied Biosystems, Warrington, UK).

VI.2.2.2 Growth conditions

For each experiment, *C. glabrata* ATCC2001 and *C. glabrata* HT6 strains were subcultured on Sabouraud dextrose agar (SDA) (Merck, Darmstadt, Germany) and the mutant *C. glabrata Δmnn2* was cultured on SD-his, as indicated by West et al [17], during 24 h at 37°C. Cells were then inoculated in Sabouraud dextrose broth (SDB) (Merck, Darmstadt, Germany) and incubated for 18 h at 37°C under agitation at 120 rpm. After incubation, the cells were harvested by centrifugation at 3000 *g* for 10 min at 4°C and washed twice with Phosphate Buffered Saline (PBS 0.1 *M*,

pH=7.5). Pellets were then suspended in RPMI-1640 (pH=7, Sigma-Aldrich, Roswell Park) and the cellular density was adjusted to $1x10^5$ cells/mL, using a Neubauer counting chamber.

VI.2.2.3Antifungal drugs

Fluconazole, caspofungin and micafungin were kindly provided by Pfizer®, S.A., MSD® and Astellas® respectively, in its pure compound. Amphotericin B was purchased in Sigma. Aliquots of 5000 mg/L were prepared using dimethyl-sulfoxide (DMSO). The final concentrations used were prepared with RPMI-1640.

VI.2.2.4Biofilm matrix structure: confocal laser scanning microscopy

The staining and confocal scanning laser microscopy (CSLM) were performed similarly to a method described previously [45]. Biofilms were formed in 24-well polystyrene microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium) [46], on plastic highly adhesive round tissue culture coverslips (13 mm diameter, Sarstedt, Germany). For this, 1000 μL of yeast cell suspension ($1x10^5$ cells/mL in RPMI-1640) were added to each well and the biofilms performed as described previously. After 24 h, 500 μL of RPMI-1640 medium was removed and an equal volume of fresh RPMI-1640 with or without the antifungal agents was added.

The staining and confocal scanning laser microscopy (CSLM) were performed similarly to a method described previously [47]. Biofilms were fixed in 4% (w/v) of paraformaldehyde (Sigma-Aldrich, St Louis, MO, USA) followed by 50% (v/v) ethanol for 10 min at room temperature and allowed to air dry. Then, to stain α-mannopyranosyl residues of the glycosylated mannoproteins (that is, mannans) of the cells they were overlayed with 25 μg/mL Concanavalin A-Alexa Fluor® 488 conjugate (ThermoFisher Scientific, Bartlesville, USA) in the dark for 10 min at room temperature [19–21]. For cell nucleus detection, the cells were stained with 100 mg/L of 4',6-diamidino-2-phenylindole (DAPI, ThermoFisher Scientific, Bartlesville, USA) for 10 min at room temperature.

After this, the preparation was washed with water and allowed to air dry. Finally, the sample was then immediately observed using the CLSM (Olympus BX61, Model FluoView 1000). Images were acquired with the program FV10-ASW 4.2 (Olympus) using a magnification of ×100.

VI.2.2.5Biofilm Antifungal susceptibility tests

The antifungal susceptibility tests were determined using the microdilution method, in accordance to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines[48,49].

The MBECs that were determined for *C. glabrata* ATCC2001 for all drugs (Flu, AmB, Csf and Mcf) were used to evaluate the biofilm susceptibility of *C. glabrata* Δ*mnn2* and the parent*, C. glabrata* HT6. For that, standardized cell suspensions (200 μL) were placed into selected wells of 96-wells polystyrene microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium). RPMI-1640 was used without cells, but with antifungal agent, as a negative control. As positive control cell suspensions were tested without antifungal agent. At 24 h, 100 μL of RPMI-1640 was removed and an equal volume of fresh RPMI-1640 plus the respective antifungal concentration were added (Flu: 1250 mg/L; AmB: 4 mg/L; Csf: 3 mg/L; Mcf: 17 mg/L - 2x concentrated). The plates were incubated at 37°C for more 24 h, a total of 48 h at 120 rpm. The number of cultivable cells on biofilms was determined by the enumeration of CFUs. For that, after the period of biofilm formation, all medium was aspired and the biofilms washed once with 200 μL of PBS to remove non-adherent cells. Then, biofilms were scraped from the wells and the suspensions were vigorously vortexed for 2 min to disaggregate cells from the matrix. Serial decimal dilutions in PBS were plated on SDA and incubated for 24 h at 37°C [50]. The results were presented in percentage of biofilm cell reduction.

Total biofilm biomass was quantified by CV staining [50]. After biofilms formation, the medium was aspirated and non-adherent cells removed by washing the biofilms with sterile ultra-pure water. Then, biofilms were fixed with 200 μL methanol, which was removed after 15 min of contact. The microtiter plates were allowed to dry at room temperature, and 200 μL of CV (1% v/v) were added to each well and incubated for 5 min. The wells were then gently washed twice with sterile, ultra-pure water and 200 μL of acetic acid (33% v/v) were added to release and dissolve the stain. The absorbance of the obtained solution was read in a microtiter plate reader (Bio-Tek Synergy HT, Izasa, Lisbon, Portugal) at 570 nm. The results were presented as absorbance per unit area (Abs/cm^2).

VI.2.2.6 Effect of the antifungals on biofilm cells' walls and on biofilm matrix composition

VI.2.2.6.1 Matrix extraction method

Biofilms were formed as explained above. After 48 h, biofilms were scraped from the 24-well plates, resuspended in ultra-pure water, sonicated (Ultrasonic Processor, Cole-Parmer, Illinois, USA) for 30 s at 30 W, and then the suspension vortexed for 2 min. The suspension was centrifuged at 5000 *g* for 5 min at 4°C and the supernatant filtered through a 0.2 μm nitrocellulose filter. The pellets were dried at 37°C until a constant dry biofilm weight was determined.

VI.2.2.6.2 Quantification of mannans on biofilm cells and on biomass – Quantitative Alcian Blue Binding Assay

Alcian Blue assay was performed according previously described by Odani et al [51] with slight adjustments [47]. Regarding the mannans on cell membranes, biofilms of *C. glabrata* strains were grown as previously described, scraped and sonicated (Ultrasonic Processor, Cole-Parmer, Illinois, USA) for 30 s at 30 W, and then the suspension vortexed for 2 min, to separate cells form the biofilm matrices. Next, the isolated cells were washed with deionized water, the OD_{600} read and adjusted at an OD_{600} of 0.6 in deionized water. Afterwards, the cells were washed with 0.02 N HCl (pH=3), the pellet was suspended in 1 mL of 30 μg/mL Alcian blue dye dissolved in 0.02 N HCl (pH=3) and incubated for 10 min at room temperature. The cells were then pelleted and evaluated for dye binding. To quantify dye binding and dye remaining in the supernatant after binding the stain was measured spectrophotometrically at 600 nm. To calculate the Alcian Blue Binding the following equations were used:

Amount of dye adsorbed to the cells (*T*): $T\ (\mu g) = 61.3 \times (OD_{600}\ ori - OD600\ sup)$ ($OD_{600}\ ori$ – OD of the Alcian Blue Dye working solution; $OD_{600}\ sup$ - OD of the supernatant);

Alcian Blue Binding (μg/OD600) = $(T \times d) / OD_{600}\ c.c.$

($OD_{c.c.}$ - the exact OD reading; d – dilution factor).

VI.2.2.6.3 Alcian Blue quantitative assay on biofilm

To measure the relative percentage of mannans on biofilm (total biomass), the medium with planktonic cells was all removed from the well and the biofilm was washed with 0.02 N HCl (pH=3). Next, the biofilm was scraped, suspended in 1 mL of 30 μg/mL Alcian blue dye dissolved in 0.02 N HCl (pH=3), incubated for 10 min at room temperature and pelleted for evaluation of dye binding. To calculate relative percentage of mannans on biofilm the following equation was used:

% mannans on biofilm: $[(OD_{600}\ ori - OD_{600}\ sup) / OD_{600}\ ori] \times 100$

($OD_{600}ori$ – OD of the Alcian Blue Dye working solution; $OD_{600}\ sup$ - OD of the supernatant);

VI.2.2.6.4 Isolation of *Candida glabrata* biofilm cells walls.

Cell wall fractionation was performed as described by Pitarch et al [52] with slight modifications. For that, biofilms were prepared as described above. *C. glabrata* biofilm cells were collected by centrifugation, washed two times with PBS and sonicated at 30 W for 30 seconds, in order to allow separation of the cells from matrix. Next, biofilm cells were separated by centrifugation at

5000 *g* for 5 min in a cell wall fraction (pellet) and a soluble cytoplasmic fraction (supernatant). Following this, the cell wall fraction was dissolved in 500 µL of buffer with phenylmethylsulfonyl fluoride (PMSF) (1mM) and transfered to "lysis" tube with 500 µL of glass beads for the cells disruption of the cells in the FastPrep® (MP, Biomedicals) at maximum speed for 30 sec. The "lysis" tube was placed in a mixture of ice and water for 30 sec. These steps were repeated seven more times. This procedure was carried out until complete cell breakage, verified beforehand by phase-contrast microscopic examinations and *a posteriori* by the failure of cells to grow on YPD-chloramphenicol plates. Then, the samples were transfered to microcentrifuge tubes and spin in microfuge centrifuge at 4°C for 3 min at 2500 g. Hereafter, the isolated cell walls were washed with solutions of decreasing concentrations of NaCl (five times with each of the following ice-cold solutions: 5% NaCl, 2% NaCl, 1% NaCl, and 1 mM PMSF) to remove any extracellular or cytosolic protein contaminants that might be adhered to the cell walls through electrostatic forces. The supernatant was transferred to fresh microfuge tubes and centrifuged at 4°C for 10 min at 10000 g. The supernatants were finally transferred to fresh tubes.

VI.2.2.6.5 β-1,3-glucans concentration determination.

The β-1,3-glucans concentration was determined using Glucatell® kit (Cape Cod®, East Falmouth, USA). The values were normalized per dry weight of biofilm on biofilm matrices and per pg/mL of β-1,3-glucans on biofilm cells.

VI.2.2.7 Statistical Analysis

The experiments were performed in triplicate and in three independent assays. The results were compared using one-way ANOVA, Tukey's post hoc multiple comparisons tests, using GraphPad™ Prism 7™ (CA, USA) software. All tests were performed with a confidence level of 95%.

VI.3.2. Results

In order to compare results, three strains of *C. glabrata* were used: the reference and wild type strain *C. glabrata* ATCC2001, and the *MNN2* gene knock out (KO) strain (*C. glabrata* Δ*mnn2*) and its parent, *C. glabrata* HT6.

First, with the purpose of visualizing the effect of the *MNN2* gene KO on *C. glabrata*, the biofilms were observed microscopically by confocal scanning laser microscopy (CLSM) (Figure VI.2.1). The images of the 48-hour-biofilms of *C. glabrata* ATCC2001, *C. glabrata* HT6 and *C. glabrata* Δ*mnn2*

showed that the gene deletion, apparently, had no effect on the cell wall, since there were no relevant differences among the three strains (Figure VI.2.1). Regarding the biofilms structure, all strains demonstrated to have good biofilm forming capacity, composed by yeasts in a continuous carpet [53,50,54] (Figure VI.2.1). Yet and curiously, *C. glabrata* Δ*mnn2* and *C. glabrata* HT6 exhibited higher amount of multilayer structures, whereas *C. glabrata* ATCC2001 showed a less amount of yeast cells forming these structures (Figure VI.2.1).

VI.3.2.1 Effect of the antifungals on biofilms

The effect of several drugs - Flu, AmB, Csf and Mcf - was tested on *C. glabrata* ATCC2001, *C. glabrata* Δ*mnn2* and *C. glabrata* HT6 biofilms. The MBECs for *C. glabrata* ATCC2001 were determined previously and used as reference value for the other strains in this study. The results of the determination of the total biomass quantification are shown in Figure VI.2.2. In general, all strains showed a biomass reduction after contact with antifungals, but the response was not linear for the three *C. glabrata* strains. The controls showed to have high capacities to produce biofilm, demonstrating that the lack of mannans in the mutant does not affect the quantity of biofilm produced. Nevertheless, when drugs are added to the 24-h-biofilm, the effect is completely altered. The mutant strain had a higher biomass loss, when compared to the controls and to *C. glabrata* ATCC2001 and *C. glabrata* HT6 (Figure VI.2.2).

Table VI.2.1 shows the CFU count of the biofilm cells. When in contact with the drugs (MBEC), both the mutant and the parent strain were more resistant to Flu, AmB, Csf and Mcf, than ATCC2001. Interestingly, only AmB was capable of reducing more than 1 Log_{10} of the initial CFU in the mutant strain, evidencing a higher resistant profile, when compared to the wild type strain, in which the echinocandins showed to have a good antifungal activity. The results demonstrate that, the initial CFU of the biofilm cells are very similar among the strains. It is, thus, probable that the differences in the antifungals susceptibilities are related to the biofilm matrices and/or in differences in the cell wall composition of each strain. In order to evaluate this, the composition of the cells walls and the biofilm matrices were also determined.

VI.3.2.2Effect of the antifungals on biofilm's cells walls and matrix

In order to estimate the phosphomannans content in the biofilm cells, the quantitative Alcian Blue binding assay was performed (Figure VI.2.3).

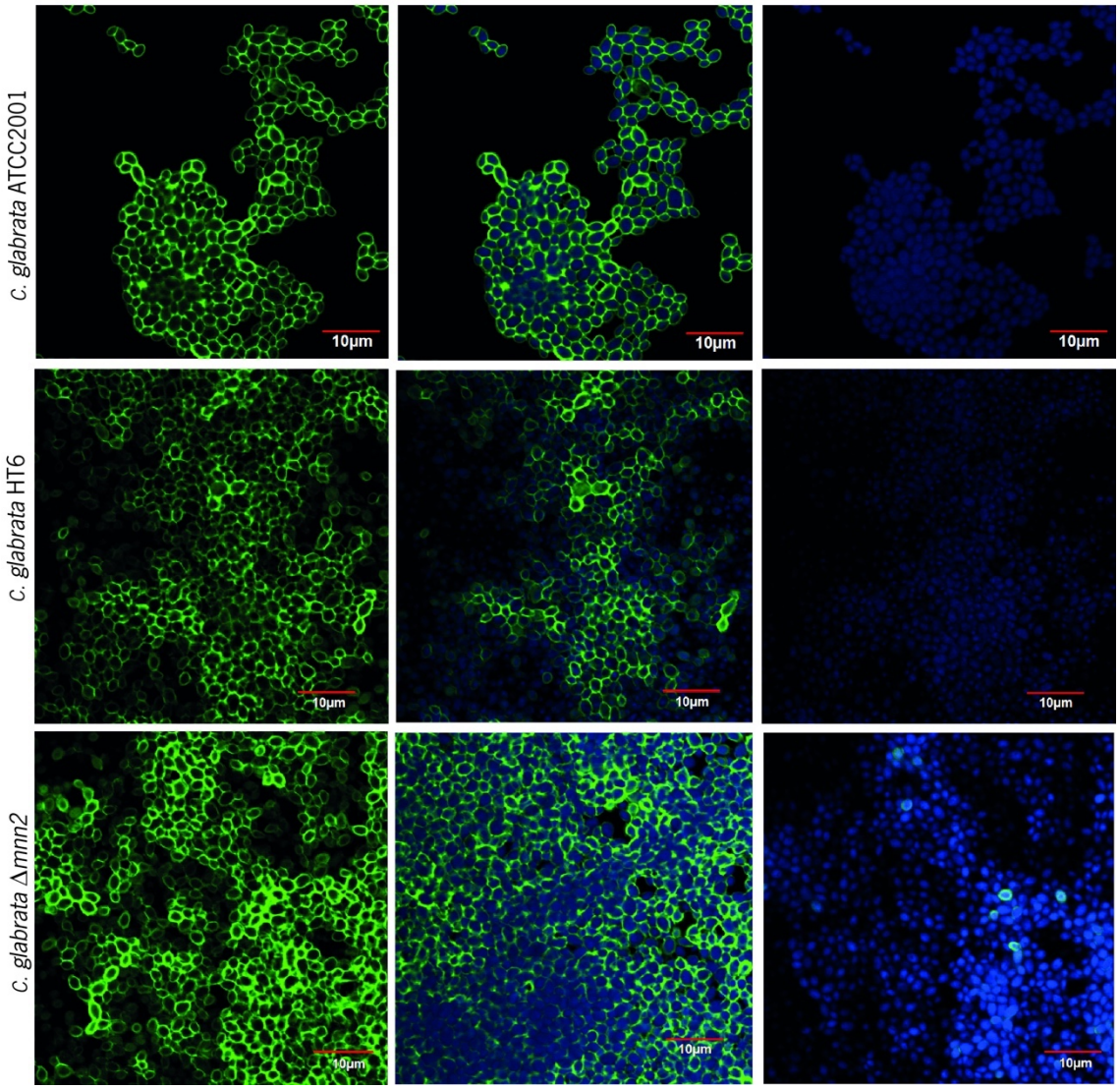

Figure VI.2.1. Confocal laser scanning microscopy image a 48-hour-biofilm of *C. glabrata* ATCC2001, *C. glabrata* HT6 and *C. glabrata* Δ*mnn2*. The biofilm images were acquired using a Confocal Scanning Laser Microscope (Olympus BX61, Model FluoView 1000). Filters: DAPI (100 mg/L emissions filters BA 430–470) and Concanavalin A, Alexa Fluor 488 conjugate (50 mg/L emissions filters BA 505–605). Images were acquired with the program FV10-ASW 4.2 (Olympus) using a magnification of 100x. Measure bar: 10 µm.

The results showed that the biofilm cells' mannans increased their concentration after contact with antifungals drugs in *C. glabrata* ATCC2001 (Figure VI.2.3) and, comparing to the control group, *C. glabrata* HT6 showed to have an increase in mannans when Flu, AmB and Mcf was applied to the biofilm, but not with Csf.

Regarding *C. glabrata* Δ*mnn2,* the performance was different. The KO of *MNN2* gene significantly reduced the ability of cells to bind Alcian Blue, in comparison with the other strains, to a point that it could not be quantified (Figure VI.2.3).

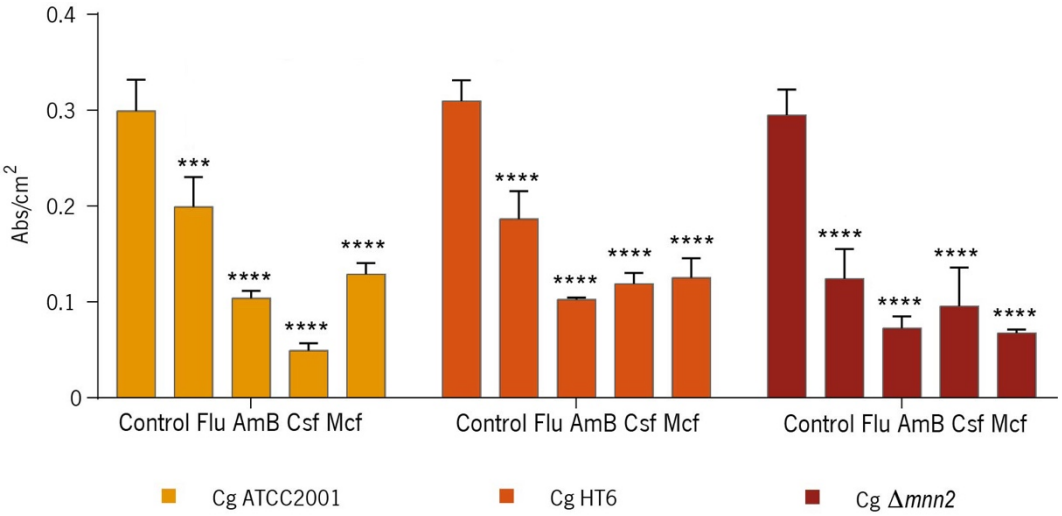

Figure VI.2.2. Crystal Violet in a 48-hour-biofilm of *C. glabrata* ATCC2001, *C. glabrata* HT6 and *C. glabrata* Δ*mnn2* with and without antifungal agents. The quantification of the biomass is presented by Abs/cm^2. *(*** P<0.0005; **** P< 0.0001).*

Table VI.2.1. Log_{10} CFU/cm^2 ± SD (standard deviation) biofilm cells using the MBECs of the reference strain (*C. glabrata* ATCC2001) for Flu, AmB, Csf and Mcf for *C. glabrata* Δ*mnn2* and *C. glabrata* HT6

Strain	Control	Flu	AmB	Csf	Mcf
C. glabrata ATCC2001	5,90 ± 0,18	4,95 ± 0,44	4,40 ± 0,30	3,80 ± 1,27	3,40 ± 0,07
C. glabrata Δ*mnn2*	6,16 ± 0,31	5,10 ± 0,43	4,23 ± 0,58	5,61 ± 0,11	4,98 ± 0,13
C. glabrata HT6	5,80 ± 0,36	3,69 ± 0,02	4,37 ± 0,45	5,42 ± 0,42	5,53 ± 0,41

SD-standard deviation

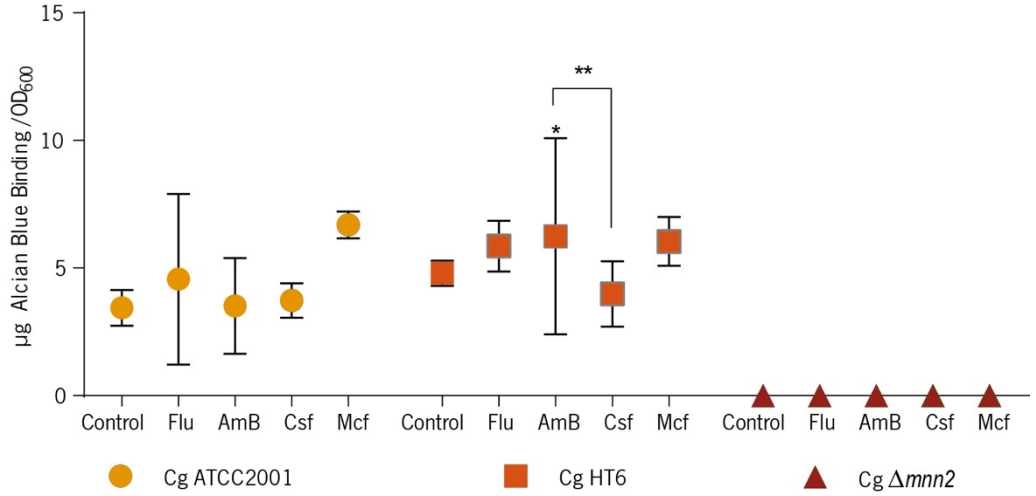

Figure VI.2.3. Alcian Blue binding assay. Data represent the mean amount of dye bound per biofim cell of *Candida glabrata* ATCC2001, *C. glabrata* HT6 and *C. glabrata* Δ*mnn2* (undetected). (Cg – *C. glabrata.* ** P<0.05; ** P<0.001).*

In addition, the content in mannans was also quantified on the biofilm matrices, which was, to authors' knowledge, performed here for the first time (Figure VI.2.4). In comparison to the controls,

the results obtained showed that all strains had less mannans on the matrices stressed by the presence of antifungals drugs. Moreover, and not surprisingly, the mutant had the less quantity of these compounds on its matrices. In average, the controls had 50 to 60% of mannans in the matrices compositions, while *C. glabrata* Δ*mnn2* had less than 20% (Figure VI.2.4). The percentage decreased about 20% after applying almost all drugs, for *C. glabrata* ATCC2001 and *C. glabrata* HT6 and approximately 10% for the mutant, except for Csf, in which condition, no mannans were detected ($P<0.0005$) (Figure VI.2.4).

The quantification of the β-1,3-glucans on the biofilm matrices of the three *C. glabrata* strains (Figure VI.2.5) showed that, comparing to the control groups, the content of β-1,3-glucans increased in the matrices of *C. glabrata* ATCC2001.

In the case of *C. glabrata* HT6, particularly with Flu 1250 mg/L ($P< 0.0001$) and AmB 4 mg/L ($P< 0.0001$) there was a rise in glucans production, statistically significant results were observed for AmB 4 mg/L and Mcf 17 mg/L ($P< 0.05$ and $P< 0.001$, respectively), for *C. glabrata* Δ*mnn2*.

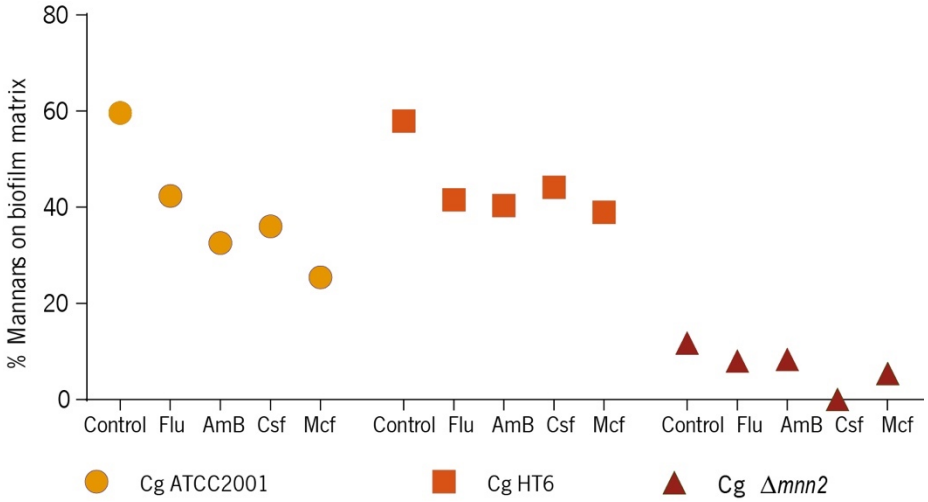

Figure VI.2.4. Alcian Blue binding assay in biofilm matrices of *C. glabrata* ATCC2001, *C. glabrata* HT6 and *C. glabrata* Δ*mnn2*. Data represent the percentage of mannans on biofilm (Cg – *C. glabrata).*

Curiously, although the mutant revealed to have less quantity of total polysaccharides in the biofilm matrix than the reference and the parent strain (data not shown), it showed to have the highest quantity of β-1,3-glucans per dry weight of biofilm matrix among all strains (7,66E+05 ± 1,03E+05), demonstrating a matrix richer in glucans' polymers than the other two strains (Figure VI.2.5).

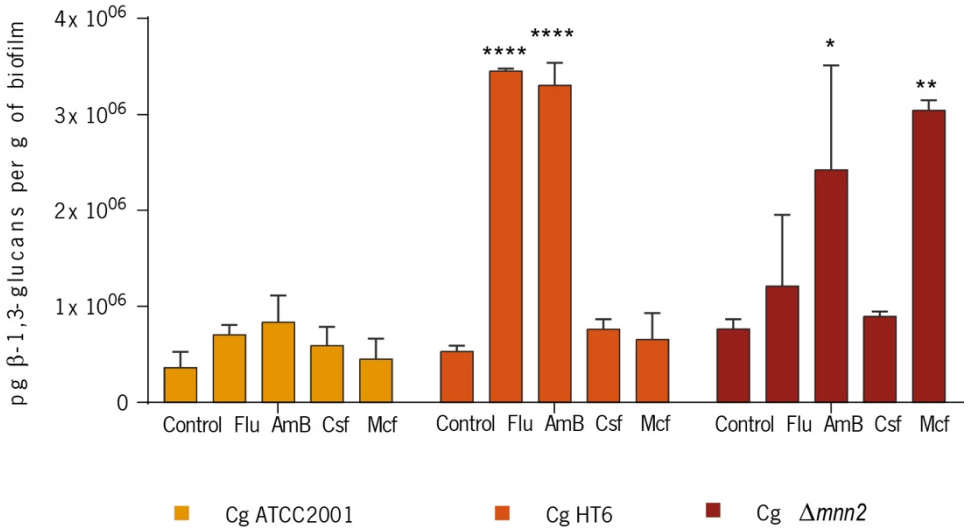

Figure VI.2.5. β-1,3-glucans concentration (pg/mL) on the biofilm matrices of *C. glabrata* ATCC2001, *C. glabrata* HT6 and *C. glabrata* Δ*mnn2*. The values were normalised per dry weight of biofilm (Cg – *C. glabrata*. ** P<0.05; ** P<0.001; *** P<0.0005; **** P< 0.0001*).

Table VI.2.2. β-1,3-glucans concentration (pg/mL) on the biofilm cells walls of *C. glabrata* ATCC2001, *C. glabrata* HT6 and *C. glabrata* Δ*mnn2 (** P<0.001)*

Strain	**β-1,3-glucans concentration on the biofilm cells walls (pg/mL) ± SD**
C. glabrata ATCC2001	359.00 ± 8.20
C. glabrata HT6	370.00 ± 5.08
C. glabrata Δ*mnn2*	387.00 ± 7.00 (*)

SD-standard deviation

Afterwards, and in order to verify if the *C. glabrata* Δ*mnn2* initial cells were compensating the lack mannans of their cell walls with glucans, it was performed the quantification of β-1,3-glucans of the biofilm cells' walls of the controls of the three strains (Table VI.2.2). The results confirmed that the content of β–1,3-glucans increased, in the mutant cell walls meaning that they have, indeed, a cell wall richer in β–1,3-glucans, when compared with HT6 (statistically significant).

VI.4.2. Discussion

Systemic candidiasis is a worldwide emergent problem [1,55], with high mortality and high economic costs associated [53,56]. The fungal cell wall consists of β-1,3-glucans, β-1,4-glucans, β -1,6-glucans and chitin [21,22] and a layer of highly glycosylated mannoproteins [19–21]. Immunologically, these proteins called phosphomannans [37] are mainly organised in mannans, having a crucial role in adhesion and host recognition (host-fungus interactions) [17,23–36].

The results on the CLSM images of the 48-hour-biofilms of all strains revealed that, microscopically, there were no variances on the cell wall among the strains (Figure VI.2.1), but the total biomass

quantification (Figure VI.2.2) showed nonlinear biomass reduction after contact with antifungals in the *C. glabrata* strains. When compared to the other two strains, *C. glabrata* Δ*mnn2* demonstrated a higher biomass loss, which was assumed to be correlated to the KO in the *MNN2* gene and subsequently, the reduced quantity of mannans in its biofilm matrix (Figure VI.2.2). Following, the susceptibility of the strains to the antifungal drugs, the results showed that the *C. glabrata* Δ*mnn2* biofilms were more resistant to Flu, AmB and both echinocandins, than *C. glabrata* ATCC2001. Adding this to the fact that the initial CFU of the biofilm cells (Table VI.2.1) was similar among the strains, led us to believe that these differences in the drugs susceptibility were related to the cell walls and/or biofilm matrices composition, similarly to previous results from our group [57–59]. Then, the biofilm matrices and cell walls were explored.

The Alcian Blue is a cationic dye, binding the negatively charged phosphate group of the phosphomannan [2,47,51]. This capacity is used to estimate the cell wall phosphomannan content [2,47,51]. The N-linked phosphomannan is attached to the branched mannan through α-1,2-mannose residues, which extend out from the α-1,6-mannose backbone. Consequently, a deletion of any the *MNN2* family members was expected to disturb the phosphomannan content of the cell wall [2]. *C. glabrata* ATCC2001 increased the quantities of mannans on its cell walls in the presence of drugs (Figure VI.2.3), which may be a possible adaptation of the cells to the stress caused by the antifungal agents. Similar cell walls adjustments during drug pressure have been described extensively as being directly responsible for antifungal resistance events [1,53,60–63]; comparing to the control group, the parent strain (*C. glabrata* HT6) increased the mannans content when stressed by Flu, AmB and Mcf, but not by Csf (statistically significant, comparing to the AmB, $P<0.001$), which demonstrated the intra-strain variability, genotypic and phenotypic alterations and also adaptable drug responses [64]. Though, *C. glabrata* Δ*mnn2* considerably reduced the ability of cells to bind Alcian Blue (Figure VI.2.3), as it was demonstrated before [17], suggesting that *MNN2* genes family are essential for phosphomannan integration, while deletion of other genes indicated a minor reduction in phosphomannan concentration [2,37]. This has been previously revealed for planktonics [2,17] and for other species [37,43,65–67]. Those results raised the possibility that other polysaccharides' metabolism (e.g. β-1,3-glucans) could be enhanced in the detriment of mannans in the mutant. Concerning the Alcian Blue binding assay in the biofilm matrices, all the *C. glabrata* strains showed to have less mannans on the matrices stressed by the presence of antifungals drugs, especially the mutant (Figure VI.2.4), which revealed that a poorer

mannans' matrix is a more fragile matrix, more sensible to environmental stresses and, thus, more susceptible to biomass loss (corroborating the biomass results).

Next, the results on the determination of the β-1,3-glucans showed that these compounds tended to increase their presence in the biofilm matrices when the biofilms were in contact with the drugs (Figure VI.2.5). It appears that, after a stress situation, the reduction in mannans that was observed earlier (Figure VI.2.4) matches the β-1,3-glucans increase in the *C. glabrata* strains (Figure VI.2.5). Moreover, the controls of the reference and the parent strain had ≈60% of mannans in the matrices compositions, while *C. glabrata* Δ*mnn2* had less than 20%. This reinforces the fact that the *C. glabrata* Δ*mnn2* biofilm matrix was mostly constituted by β-glucans, as it was determined (Figure VI.2.5). The quantification of β-1,3-glucans of the cells walls of the biofilm cells of the strains (Table VI.2.2) confirmed that the quantity of these biopolymers is variable in the cell walls of *C. glabrata* strains. The mutant displayed a cell wall richer in β-1,3-glucans, when compared to the others (statistically significant), which demonstrates higher capacities of compensation mechanisms, which, adding to the increase in the β-1,3-glucans content of the matrix, may be responsible for a more resistance profile to the drugs (Table VI.2.2).

VI.5.2. Conclusion

The strong variability in the biofilm matrices and in the cell walls composition of the *C. glabrata* strains has been formerly stated after any drug pressure, being related to higher pathogenicity and virulence states. Though, other factors are related to the biofilm drug resistance and one single feature in not able to clarify the complete phenomenon of resistance [50,53,57,68]. The KO of the *MNN2* gene has demonstrated to influence the drug response profile of biofilm cells, by inducing key changes in the matrices and cell wall's compositions, validating again the great importance of β-glucans in the resistance of *C. glabrata* biofilms to antifungals. Finally, this work also suggests that the identification and blocking of genes directly related to the plasticity of the composition of the matrices and cell walls are a good path to the search to new antifungal agents.

References

1. Rodrigues, C. F.; Rodrigues, M. E.; Silva, S.; Henriques, M. Candida glabrata Biofilms: How Far Have We Come? *J. Fungi* **2017**, *3*, 11, doi:10.3390/JOF3010011.

2. Hall, R. A.; Bates, S.; Lenardon, M. D.; Maccallum, D. M.; Wagener, J.; Lowman, D. W.; Kruppa, M. D.; Williams, D. L.; Odds, F. C.; Brown, A. J. P.; Gow, N. A. R. The Mnn2 Mannosyltransferase Family Modulates Mannoprotein Fibril Length, Immune Recognition and Virulence of Candida albicans. *PLoS Pathog* **2013**, *9*, doi:10.1371/journal.ppat.1003276.

3. Almirante, B.; Rodríguez, D.; Park, B. J.; Cuenca-Estrella, M.; Planes, A. M.; Almela, M.; Mensa, J.; Sanchez, F.; Ayats, J.; Gimenez, M.; Saballs, P.; Fridkin, S. K.; Morgan, J.; Rodriguez-Tudela, J. L.; Warnock, D. W.; Pahissa, A.; Rodrı, D. Epidemiology and Predictors of Mortality in Cases of Candida Bloodstream Infection : Results from Population-

Based Surveillance , Barcelona , Spain , from 2002 to 2003 Epidemiology and Predictors of Mortality in Cases of Candida Bloodstream Infection : Re. *J. Clin. Microbiol.* **2005**, *43*, 1829–1835, doi:10.1128/JCM.43.4.1829.
4. Colombo, A. L.; Guimaraes, T.; Silva, L. R. B. F.; Monfardini, L. P. de A.; Cunha, A. K. B.; Rady, P.; Alves, T.; Rosas, R. C. Prospective Observational Study of Candidemia in Sao Paulo, Brazil: Incidence Rate, Epidemiology, and Predictors of Mortality • . *Infect. Control Hosp. Epidemiol.* **2007**, *28*, 570–576, doi:10.1086/513615.
5. Bassetti, M.; Righi, E.; Costa, A.; Fasce, R.; Molinari, M. P.; Rosso, R.; Pallavicini, F. B.; Viscoli, C. Epidemiological trends in nosocomial candidemia in intensive care. *BMC Infect. Dis.* **2006**, *6*, 21, doi:10.1186/1471-2334-6-21.
6. Hasan, F.; Xess, I.; Wang, X.; Jain, N.; Fries, B. C. Biofilm formation in clinical Candida isolates and its association with virulence. *Microbes Infect.* **2009**, *11*, 753–61, doi:10.1016/j.micinf.2009.04.018.
7. Sardi, J. C. O.; Scorzoni, L.; Bernardi, T.; Fusco-Almeida, A. M.; Mendes Giannini, M. J. S. Candida species: Current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. *J. Med. Microbiol.* **2013**, *62*, 10–24, doi:10.1099/jmm.0.045054-0.
8. Lim, C. S.-Y.; Rosli, R.; Seow, H. F.; Chong, P. P. Candida and invasive candidiasis: back to basics. *Eur. J. Clin. Microbiol. Infect. Dis.* **2012**, *31*, 21–31, doi:10.1007/s10096-011-1273-3.
9. Ruan, S.-Y.; Lee, L.-N.; Jerng, J.-S.; Yu, C.-J.; Hsueh, P.-R.; Saral, R. Candida glabrata fungaemia in intensive care units. *Clin. Microbiol. Infect.* **2008**, *14*, 136–40, doi:10.1111/j.1469-0691.2007.01892.x.
10. Wisplinghoff, H.; Seifert, H.; Tallent, S. M.; Bischoff, T.; Wenzel, R. P.; Edmond, M. B. Nosocomial bloodstream infections in pediatric patients in United States hospitals: epidemiology, clinical features and susceptibilities. *Pediatr. Infect. Dis. J.* **2003**, *22*, 686–691, doi:10.1097/01.inf.0000078159.53132.40.
11. Abi-Said, D.; Anaissie, E.; Uzun, O.; Pinzcowski, H.; Vartivarian, S. The epidemiology of hematogeneous candidiasis caused by different Candida species. *Clin Infect Dis* **1997**, *24*, 1122–28.
12. Pfaller, M.; Jones, R.; Messer, S.; Edmond, M.; Wenzel, R. National surveillance of nosocomial blood stream infection due to species of Candida other than Candida albicans: frequency of occurrence and antifungal susceptibility in the SCOPE Program. *Diagn Microbiol Infect Dis* **1998**, *30*, 121–29.
13. Pfaller, M. A.; Jones, R. N.; Doern, G. V; Sader, H. S.; Messer, S. A.; Houston, A.; Coffman, S.; Hollis, R. J. Bloodstream infections due to Candida species: SENTRY antimicrobial surveillance program in North America and Latin America, 1997–1998. *Antimicrob Agents Chemother* **2000**, *44*, 747–51.
14. Gumbo, T.; Isada, C.; Hall, G.; Karafa, M.; Gordon, S. Candida glabrata fungemia. Clinical features of 139 patients. *Med.* **1999**, *78*, 220–27.
15. Pfaller, M.; Jones, R.; Doern, G.; Fluit, A.; Verhoef, J.; Sader, H.; Messer, S.; Houston, A.; Coffman, S.; Hollis, R. International surveillance of blood stream infections due to Candida species in the European SENTRY Program: species distribution and antifungal susceptibility including the investigational triazole and echinocandin agents. SENTRY Participant Group (Euro). *Diagn Microbiol Infect Dis* **1999**, *35*, 19–25.
16. Costa-Orlandi, C.; Sardi, J.; Pitangui, N.; de Oliveira, H.; Scorzoni, L.; Galeane, M.; Medina-Alarcón, K.; Melo, W.; Marcelino, M.; Braz, J.; Fusco-Almeida, A.; Mendes-Giannini, M. Fungal Biofilms and Polymicrobial Diseases. *J. Fungi* **2017**, *3*, 22, doi:10.3390/jof3020022.
17. West, L.; Lowman, D.; Mora-Montes, H.; Grubb, S.; Murdoch, C.; Thornhill, M.; Gow, N.; Williams, D.; Haynes, K. Differential virulence of Candida glabrata glycosylation mutants. *Biol Chem.* **2013**, *288*, 22006–18.
18. Klis, F. M.; Boorsma, A.; De Groot, P. W. J. Cell wall construction inSaccharomyces cerevisiae. *Yeast* **2006**, *23*, 185–202, doi:10.1002/yea.1349.
19. Ruiz-Herrera, J.; Victoria Elorza, M.; Valent??n, E.; Sentandreu, R. Molecular organization of the cell wall of *Candida albicans* and its relation to pathogenicity. *FEMS Yeast Res.* **2006**, *6*, 14–29, doi:10.1111/j.1567-1364.2005.00017.x.
20. Latg, J.-P. The cell wall: a carbohydrate armour for the fungal cell. *Mol. Microbiol.* **2007**, *66*, 279–290, doi:10.1111/j.1365-2958.2007.05872.x.
21. Netea, M. G.; Brown, G. D.; Kullberg, B. J.; Gow, N. A. R. An integrated model of the recognition of Candida albicans by the innate immune system. *Nat. Rev. Microbiol.* **2008**, *6*, 67–78, doi:10.1038/nrmicro1815.
22. Bowman, S. M.; Free, S. J. The structure and synthesis of the fungal cell wall. *BioEssays* **2006**, *28*, 799–808, doi:10.1002/bies.20441.
23. Netea, M. G.; Mardi, L. Innate immune mechanisms for recognition and uptake of Candida species. *Trends Immunol.* **2010**, *31*, 346–353, doi:10.1016/j.it.2010.06.007.
24. Gow, N. A. R.; Netea, M. G.; Munro, C. A.; Ferwerda, G.; Bates, S.; Mora-Montes, H. M.; Walker, L.; Jansen, T.; Jacobs, L.; Tsoni, V.; Brown, G. D.; Odds, F. C.; Van der Meer, J. W. M.; Brown, A. J. P.; Kullberg, B. J. Immune recognition of Candida albicans beta-glucan by dectin-1. *J. Infect. Dis.* **2007**, *196*, 1565–71, doi:10.1086/523110.
25. van de Veerdonk, F. L.; Kullberg, B. J.; van der Meer, J. W.; Gow, N. A.; Netea, M. G. Host?microbe interactions: innate pattern recognition of fungal pathogens. *Curr. Opin. Microbiol.* **2008**, *11*, 305–312, doi:10.1016/j.mib.2008.06.002.
26. Reid, D. M.; Gow, N. A.; Brown, G. D. Pattern recognition: recent insights from Dectin-1. *Curr. Opin. Immunol.*

2009, *21*, 30–37, doi:10.1016/j.coi.2009.01.003.
27. Mora-Montes, H. M.; Bates, S.; Netea, M. G.; Castillo, L.; Brand, A.; Buurman, E. T.; Diaz-Jimenez, D. F.; Jan Kullberg, B.; Brown, A. J. P.; Odds, F. C.; Gow, N. A. R. A Multifunctional Mannosyltransferase Family in Candida albicans Determines Cell Wall Mannan Structure and Host-Fungus Interactions. *J. Biol. Chem.* **2010**, *285*, 12087–12095, doi:10.1074/jbc.M109.081513.
28. Taylor, P. R.; Tsoni, S. V.; Willment, J. A.; Dennehy, K. M.; Rosas, M.; Findon, H.; Haynes, K.; Steele, C.; Botto, M.; Gordon, S.; Brown, G. D. Dectin-1 is required for ?-glucan recognition and control of fungal infection. *Nat. Immunol.* **2007**, *8*, 31–38, doi:10.1038/ni1408.
29. Murciano, C.; Moyes, D. L.; Runglall, M.; Islam, A.; Mille, C.; Fradin, C.; Poulain, D.; Gow, N. A. R.; Naglik, J. R. Candida albicans Cell Wall Glycosylation May Be Indirectly Required for Activation of Epithelial Cell Proinflammatory Responses. *Infect. Immun.* **2011**, *79*, 4902–4911, doi:10.1128/IAI.05591-11.
30. Gow, N. A.; Hube, B. Importance of the Candida albicans cell wall during commensalism and infection. *Curr. Opin. Microbiol.* **2012**, *15*, 406–412, doi:10.1016/j.mib.2012.04.005.
31. Bates, S.; Hughes, H. B.; Munro, C. A.; Thomas, W. P. H.; MacCallum, D. M.; Bertram, G.; Atrih, A.; Ferguson, M. A. J.; Brown, A. J. P.; Odds, F. C.; Gow, N. A. R. Outer Chain N-Glycans Are Required for Cell Wall Integrity and Virulence of Candida albicans. *J. Biol. Chem.* **2006**, *281*, 90–98, doi:10.1074/jbc.M510360200.
32. Bates, S.; MacCallum, D. M.; Bertram, G.; Munro, C. A.; Hughes, H. B.; Buurman, E. T.; Brown, A. J. P.; Odds, F. C.; Gow, N. A. R. Candida albicans Pmr1p, a Secretory Pathway P-type Ca2+/Mn2+-ATPase, Is Required for Glycosylation and Virulence. *J. Biol. Chem.* **2005**, *280*, 23408–23415, doi:10.1074/jbc.M502162200.
33. Mora-Montes, H. M.; Bates, S.; Netea, M. G.; Diaz-Jimenez, D. F.; Lopez-Romero, E.; Zinker, S.; Ponce-Noyola, P.; Kullberg, B. J.; Brown, A. J. P.; Odds, F. C.; Flores-Carreon, A.; Gow, N. A. R. Endoplasmic Reticulum -Glycosidases of Candida albicans Are Required for N Glycosylation, Cell Wall Integrity, and Normal Host-Fungus Interaction. *Eukaryot. Cell* **2007**, *6*, 2184–2193, doi:10.1128/EC.00350-07.
34. Munro, C. A.; Bates, S.; Buurman, E. T.; Hughes, H. B.; MacCallum, D. M.; Bertram, G.; Atrih, A.; Ferguson, M. A. J.; Bain, J. M.; Brand, A.; Hamilton, S.; Westwater, C.; Thomson, L. M.; Brown, A. J. P.; Odds, F. C.; Gow, N. A. R. Mnt1p and Mnt2p of Candida albicans Are Partially Redundant ?-1,2-Mannosyltransferases That Participate in O-Linked Mannosylation and Are Required for Adhesion and Virulence. *J. Biol. Chem.* **2005**, *280*, 1051–1060, doi:10.1074/jbc.M411413200.
35. Saijo, S.; Ikeda, S.; Yamabe, K.; Kakuta, S.; Ishigame, H.; Akitsu, A.; Fujikado, N.; Kusaka, T.; Kubo, S.; Chung, S.; Komatsu, R.; Miura, N.; Adachi, Y.; Ohno, N.; Shibuya, K.; Yamamoto, N.; Kawakami, K.; Yamasaki, S.; Saito, T.; Akira, S.; Iwakura, Y. Dectin-2 Recognition of α-Mannans and Induction of Th17 Cell Differentiation Is Essential for Host Defense against Candida albicans. *Immunity* **2010**, *32*, 681–691, doi:10.1016/j.immuni.2010.05.001.
36. Dean, N. Asparagine-linked glycosylation in the yeast Golgi. *Biochim. Biophys. Acta* **1999**, *1426*, 309–22.
37. Hall, R. A.; Gow, N. A. R. Mannosylation in candida albicans: Role in cell wall function and immune recognition. *Mol. Microbiol.* **2013**, *90*, 1147–1161, doi:10.1111/mmi.12426.
38. Takahashi, S.; Kudoh, A.; Okawa, Y.; Shibata, N. Significant differences in the cell-wall mannans from three Candida?glabrata strains correlate with antifungal drug sensitivity. *FEBS J.* **2012**, *279*, 1844–1856, doi:10.1111/j.1742-4658.2012.08564.x.
39. Kobayashi, H.; Mitobe, H.; Takahashi, K.; Yamamoto, T.; Shibata, N.; Suzuki, S. Structural study of a cell wall mannan-protein complex of the pathogenic yeast Candida glabrata IFO 0622 strain. *Arch. Biochem. Biophys.* **1992**, *294*, 662–9.
40. Kobayashi, H.; Oyamada, H.; Iwadate, N.; Suzuki, H.; Mitobe, H.; Takahashi, K.; Shibata, N.; Suzuki, S.; Okawa, Y. Structural and immunochemical characterization of beta-1,2-linked mannobiosyl phosphate residue in the cell wall mannan of Candida glabrata. *Arch. Microbiol.* **1998**, *169*, 188–94.
41. Okawa, Y.; Goto, K. Disappearance of antigenic factor 6 in Candida glabrata IFO 0622 strain cells cultured at high temperature. *Biol. Pharm. Bull.* **2006**, *29*, 187–9.
42. Southard, S. B.; Specht, C. A.; Mishra, C.; Chen-Weiner, J.; Robbins, P. W. Molecular analysis of the Candida albicans homolog of Saccharomyces cerevisiae MNN9, required for glycosylation of cell wall mannoproteins. *J. Bacteriol.* **1999**, *181*, 7439–48.
43. Rayner, J. C.; Munro, S. Identification of the MNN2 and MNN5 mannosyltransferases required for forming and extending the mannose branches of the outer chain mannans of Saccharomyces cerevisiae. *J. Biol. Chem.* **1998**, *273*, 26836–43, doi:10.1074/JBC.273.41.26836.
44. Williams, D. W.; Wilson, M. J.; Lewis, M. A. O.; Potts, A. J. C. Identification of Candida Species by PCR and Restriction Fragment Length Polymorphism Analysis of Intergenic Spacer Regions of Ribosomal DNA. *J. Clin. Microbiol.* **1995**, *33*, 2476–2479.
45. Zhang, S. Q.; Zou, Z.; Shen, H.; Shen, S. S.; Miao, Q.; Huang, X.; Liu, W.; Li, L. P.; Chen, S. M.; Yan, L.; Zhang, J. D.; Zhao, J. J.; Xu, G. T.; An, M. M.; Jiang, Y. Y. Mnn10 Maintains Pathogenicity in Candida albicans by Extending α-1,6-Mannose Backbone to Evade Host Dectin-1 Mediated Antifungal Immunity. *PLoS Pathog* **2016**, *12*, 1–30,

doi:10.1371/journal.ppat.1005617.
46. Silva, S.; Negri, M.; Henriques, M.; Oliveira, R.; Williams, D.; Azeredo, J. Silicone colonization by non- Candida albicans Candida species in the presence of urine. *J. Med. Microbiol.* **2010**, 747–754, doi:10.1099/jmm.0.017517-0.
47. Singleton, D. R.; Masuoka, J.; Hazen, K. C. Surface Hydrophobicity Changes of Two. *Society* **2005**, *4*, 639–648, doi:10.1128/EC.4.4.639.
48. Arendrup, M. C.; Arikan, S.; Barchiesi, F.; Bille, J.; Dannaoui, E.; Denning, D. W.; Donnelly, J. P.; Fegeler, W.; Moore, C.; Richardson, M.; Schmalreck, A.; Velegraki, A.; Verweij, P. EUCAST Technical Note on the method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia – forming moulds. *ESCMID Tech. Notes* **2008**, *14*, 982–984, doi:10.1111/j.1469-0691.2008.02086.x.
49. EUCAST *Breakpoint tables for interpretation of MICs, Version 8.1, valid from 2017-03-01*;
50. Fonseca, E.; Silva, S.; Rodrigues, C.F.; Alves, C.T.; Azeredo, J.; Henriques, M. Effects of fluconazole on Candida glabrata biofilms and its relationship with ABC transporter gene expression. *Biofouling* **2014**, *30*, 447–57, doi:10.1080/08927014.2014.886108.
51. Odani, T.; Shimma, Y. I.; Wang, X. H.; Jigami, Y. Mannosylphosphate transfer to cell wall mannan is regulated by the transcriptional level of the MNN4 gene in Saccharomyces cerevisiae. *FEBS Lett.* **1997**, *420*, 186–190, doi:10.1016/S0014-5793(97)01513-5.
52. Pitarch, A.; Sánchez, M.; Nombela, C.; Gil, C. Sequential fractionation and two-dimensional gel analysis unravels the complexity of the dimorphic fungus Candida albicans cell wall proteome. *Mol. Cell. Proteomics* **2002**, *1*, 967–82, doi:10.1074/MCP.M200062-MCP200.
53. Silva, S.; Rodrigues, C.F.; Araújo, D.; Rodrigues, M.E.; Henriques, M. Candida Species Biofilms' Antifungal Resistance. *J. Fungi* **2017**, *3*, 8, doi:10.3390/jof3010008.
54. Hawser, S. P.; Douglas, L. J. Resistance of Candida albicans biofilms to antifungal agents in vitro. *Antimicrob. Agents Chemother.* **1995**, *39*, 2128–2131.
55.Cataldi, V.; Di Campli, E.; Fazii, P.; Traini, T.; Cellini, L.; Di Giulio, M. *Candida* species isolated from different body sites and their antifungal susceptibility pattern: Cross-analysis of *Candida albicans* and *Candida glabrata* biofilms. *Med. Mycol.* **2016**, myw126, doi:10.1093/mmy/myw126.
56. Pfaller, M. A.; Castanheira, M. Nosocomial candidiasis: Antifungal stewardship and the importance of rapid diagnosis. *Med. Mycol.* 2016, *54*.
57. Rodrigues, C.F.; Henriques, M. Oral mucositis caused by Candida glabrata biofilms: failure of the concomitant use of fluconazole and ascorbic acid. *Ther. Adv. Infect. Dis.* **2017**, *1*, doi:10.1177/.
58. Rodrigues, C. F.; Gonçalves, B.; Rodrigues, M. E.; Silva, S.; Azeredo, J.; Henriques, M. The Effectiveness of Voriconazole in Therapy of Candida glabrata's Biofilms Oral Infections and Its Influence on the Matrix Composition and Gene Expression. *Mycopathologia* **2017**, *182*, 653–664, doi:10.1007/s11046-017-0135-7.
59. Rodrigues, C. F.; Silva, S.; Azeredo, J.; Henriques, M. Candida glabrata's recurrent infections: biofilm formation during Amphotericin B treatment. *Lett. Appl. Microbiol.* **2016**, *63*, 77–81, doi:10.1111/lam.12600.
60. Chen, K.-H.; Miyazaki, T.; Tsai, H.-F.; Bennett, J. E. The bZip transcription factor Cgap1p is involved in multidrug resistance and required for activation of multidrug transporter gene CgFLR1 in Candida glabrata. *Gene* **2007**, *386*, 63–72, doi:10.1016/j.gene.2006.08.010.
61. Taff, H. T.; Nett, J. E.; Zarnowski, R.; Ross, K. M.; Sanchez, H.; Cain, M. T.; Hamaker, J.; Mitchell, A. P.; Andes, D. R. A Candida biofilm-induced pathway for matrix glucan delivery: implications for drug resistance. *PLoS Pathog.* **2012**, *8*, e1002848, doi:10.1371/journal.ppat.1002848.
62. Ferrari, S.; Sanguinetti, M.; De Bernardis, F.; Torelli, R.; Posteraro, B.; Vandeputte, P.; Sanglard, D. Loss of mitochondrial functions associated with azole resistance in Candida glabrata results in enhanced virulence in mice. *Antimicrob. Agents Chemother.* **2011**, *55*, 1852–60, doi:10.1128/AAC.01271-10.
63. Mathé, L.; Van Dijck, P. Recent insights into Candida albicans biofilm resistance mechanisms. *Curr. Genet.* **2013**, *59*, 251–264.
64. Fidel, P. L.; Vazquez, J. A.; Sobel, J. Candida glabrata: Review of Epidemiology, Pathogenesis, and Clinical Disease with Comparison to C. albicans. *Clin. Microbiol. Rev.* **1999**, *12*, 80–96, doi:10.4161/viru.27373.
65. Gow, N. A. R. Components of the fungal cell wall that turn on and turn off the immune inflammatory response.
66. Ballou, L.; Cohen, R. E.; Ballou, C. E. Saccharomyces cerevisiae Mutants That Make Mannoproteins with a truncated Carbohydrate Outer Chain*. *J. Biol. Chem.* **1980**, *255*, 5986–5991.
67. Murciano, C.; Moyes, D. L.; Runglall, M.; Islam, A.; Mille, C.; Fradin, C.; Poulain, D.; Gow, N. A. R.; Naglik, J. R. The mnn2 mutant of Saccharomyces cerevisiae is a¡ected in phosphorylation of N-linked oligosaccharides. *Infect. Immun.* **2011**, *79*, 4902–4911, doi:10.1128/IAI.05591-11.
68. Jabra-rizk, M. A.; Falker, W. A.; Meiller, T. F. Fungal Biofilms and Drug Resistance. *Emerg. Infect* **2004**, *10*, 19, doi:10.1007/5584.

VII

Chapter VII

Candida glabrata infection characterization and immune response evaluation

The work presented in this chapter was adapted from:

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VII

Inflammatory cell recruitment in *Candida glabrata* biofilm cells-infected mice receiving antifungal chemotherapy

ABSTRACT

Due to a high rate of antifungal resistance, *Candida glabrata* is one of the most prevalent *Candida* spp. linked to systemic candidiasis, which is particularly critical in immunosuppressed patients. The goal of this work was to evaluate the effectiveness of two echinocandins – caspofungin (Csf) and micafungin (Mcf) - in the treatment of hematogenously disseminated infection established with *C. glabrata* biofilm cells.

CD1 mice were infected with 48 h-biofilm cells of *C. glabrata* and then treated with Csf or Mcf. After 72h, the efficacy of each drug was evaluated assessing organ fungal burden through CFU counting. Moreover, the immune cell recruitment into target organs was evaluated by flow cytometry or histopathology analysis. Fungal burden was detected higher in the liver than in the kidneys. However, none of the drugs was effective in eradicating completely *C. glabrata* biofilm cells. At the evaluated time point, flow cytometry analysis, showed a predominant mononuclear response in the spleen, which was also evident in liver and kidneys of the infected mice, as observed by histopathology analysis.

Keywords: *Candida glabrata*, candidaemia, echinocandins, resistance, biofilms, infection, micafungin, caspofungin.

VII.1 Introduction

Candida glabrata is one of the most common causes of systemic fungal infection (candidaemia), surpassed only by *Candida albicans* [1–3]. It is the second most common isolated yeast in the United States of Amrica and the third in Europe after *Candida parapsilosis*, accounting for 20% of candidaemia [reviewed in 2, 4]. As a commensal yeast, *C. glabrata* colonizes and adapts to many different niches in the human body and can be isolated from the mucosae of healthy individuals [2,5]. Yet, as an opportunistic pathogen, this fungus can also originate mucosal infections and severe candidaemia. Its biofilm forming ability and capacity to rapidly acquire resistance to antifungals (especially to azoles) [2,5,6], which in many cases can be further increased by genetic and genomic mutations (e.g. polymorphisms, formation of new cromossomes, karyotype variations) [7–9] may contribute to increased virulence.

Risk factors for the development of invasive *C. glabrata* infections in human patients comprise immunosuppression states (e.g. cancer chemotherapy, HIV infection, neutropenia), mucosal colonization by *Candida* spp., the use of indwelling medical devices (e.g. vascular catheters) and gastrointestinal surgery [10–12].

During infection, *C. glabrata* virtually colonises all organs, which reveals a high capacity to adapt to the many different niches inside the human host [1]. Systemic *C. glabrata* infections have high associated mortality [13,14] and the rise in the incidence infections caused by this yeast is to some extent attributable to its ability to tolerate or resist many antifungals commonly used in clinical practice [2,15,16]. In contrast, experimental intravenous infection of laboratory animals with *C. glabrata* do not usually cause mortality [1].

Because of the high probability of innate resistance to fluconazole, echinocandins are recommended as first-line therapy against *C. glabrata* candidaemia [17]. However, and worryingly, *C. glabrata* is the first *Candida* spp. for which resistance to echinocandins has been identified and described [18,19]. Recently, case reports of echinocandin-resistant *C. glabrata* subsequent to different echinocandin therapies are becoming more common [20–26]. Indeed, one-third of those isolates may be multidrug resistant [27] and have specific mutations in one of two "hot spot" regions of the *FKS1* or *FKS2* genes, which encode a subunit of the β-1,3-D glucan synthase protein, target of the echinocandins [20,28–30].

Biofilm cells are known to be much more resistant to antifungal treatment than planktonic cells, and responsible for severe infections [2,31–33]. Therefore, in this work, CD1 mice were infected with 48-h-biofilm cells of wild type *C. glabrata* strain ATCC2001, and then treated with the

echinocandins caspofungin (Csf) and micafungin (Mcf), in order to evaluate the organ fungal burden after 72h, the efficacy of each drug after two administrations and the associated inflammatory response.

VII.2 Material and Methods

VII.2.1 Ethics Statement

This study was performed in strict accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123), the 86/609/EEC directive and Portuguese rules (DL 129/92). All experimental protocols were approved by the competent national authority (Direcção-Geral de Veterinária), document 0420/000/000/2010. Mice Female CD1 mice, 8–12 weeks old, were purchased from Charles River (Barcelona, Spain) and kept under specific pathogen-free conditions at the Animal Facility of the Instituto de Ciências Biomédicas Abel Salazar, Porto, Portugal. Mice were maintained in individually ventilated cages (5 animals per cage) with corncob bedding, and under controlled conditions of temperature (21 ± 1 °C), relative humidity (between 45 and 65%) and light (12 h light/dark cycle). Mice had *ad libitum* access to food and water. Hiding and nesting materials were provided for enrichment. All procedures such cage changing, water and food supply, as well as intravenous and peritoneal injections were always performed during the day cycle (between 7 and 19 h).

VII.2.2 Organisms and growth conditions

One strain of the American Type Culture Collection (ATCC), *C. glabrata* ATCC2001 was subcultured on Sabouraud dextrose agar (SDA) (Merck, Darmstadt, Germany) for 24 h at 37°C. Cells were then inoculated in Sabouraud dextrose broth (SDB) (Merck, Darmstadt, Germany) and incubated for 18 h at 37°C under agitation at 120 rpm. Biofilms were formed in 24-well polystyrene microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium) [34]. For this, 1000 µL of the yeast cell suspension ($1x10^5$ cells/mL) was added to each well incubated for 24h. After 24 h, 500 µL of RPMI-1640 (Sigma-Aldrich, Roswell Park) was removed and an equal volume of fresh medium was carefully added and biofilms allowed to grow, under the same temperature and agitation conditions, for additional 24 h. After 48 h, biofilms were scraped from the 24-well plates, resuspended in ultra-pure water, sonicated (Ultrasonic Processor, Cole-Parmer, Illinois, USA) for 30 s at 30 W, and then the suspension vortexed for 2 min. The suspension was centrifuged at 5000 *g* for 5 min at 4°C,

as optimized before [34,35]. The pellets of the biofilm cells were then suspended in RPMI-1640 and the cellular density was adjusted to $5x10^8$ cells/mL, using a Neubauer counting chamber.

VII. 2.3 Antifungal drugs

Csf and Mcf were kindly provided by MSD® and Astellas Pharma, Inc., respectively. Aliquots of 5000 mg/L were prepared using dimethyl-sulfoxide (DMSO). The final concentrations used were prepared with apyrogenic PBS for both drugs.

VII. 2.4 Murine model of hematogenously disseminated infection

The *C. glabrata* inoculum was prepared following previously described procedures [35,36]. The number of cultivable cells was assessed by CFU counting. were injected intravenously in the lateral tail vein, with the support of a restrainer, Sample size was determined based on the results of preliminary experiments. It was not possible to perform subsequent mouse studies in a blinded fashion.

On day 0, adult CD1 mice, weighting 26 to 40 g, randomly allocated to each experimental group, mice received 200 μL of the suspension of *C. glabrata* containing $5x10^8$ CFU intravenously (i.v.) via the tail vein (biofilm cell suspensions). Control mice were injected intravenously with 200 μL of apyrogenic PBS. Treatment with an echinocandin started 24 h postinoculation and was administered intraperitoneally (i.p.) in a 0.5 mL volume at 24 and 48 h postinoculation. Doses were as follows: caspofungin 6 mg/kg; and micafungin 12 mg/kg. This experimental scheme (days and dosages) were chosen on the basis of previous pharmacodynamic studies of echinocandins against *C. glabrata* and a need to reach drug exposures in mice that were comparable to those in humans receiving currently licensed echinocandin regimens [17,37,38]. Two independent experiments were performed, with at least 5 animals per infected group.

VII.2.4 Flow cytometry

For flow cytometry analysis, spleens from infected mice and controls were aseptically removed 72 h postinfection, homogenised in HBSS (Sigma-Aldrich, Roswell Park) and, when necessary, red blood cells were lysed. The following mAb were used (at previously determined optimal dilutions) for surface antigen staining after pre-incubation with anti-mouse CD16/CD32 for FcγR blocking. For dead cell exclusion, all samples except single-stained controls, were first incubated with allophycocyanin (APC) eFluor 780 Fixable Viability Dye (eBioscience, San Diego, CA) diluted 1:1000

in PBS for 30 min at 4°C. For surface staining, cells were incubated with the following monoclonal antibodies: anti-mouse GR1 Fluorescein isothiocyanate (FITC) conjugate; anti-mouse CD80 Phycoerythrin (PE) conjugate; anti-mouse F4/80 PerCp Cy5.5; anti-mouse CD86 PE-cychrome 7 (PE-Cy7)-conjugate; anti-mouse CD11c BV421-conjugate (all from BD Biosciences, San Jose, CA, USA); anti-mouse CD11b BV510-conjugate; and anti-mouse MHC class II APC conjugate (eBiosciences, San Diego, CA, USA). Data acquisition was performed in a FACSCantoTM II system (BD Biosciences, San Jose, CA) using the FACSDIVATM software (BD) and compensated and analysed in FLOWJO version 9.7.5. (Tree Star Inc., Ashland, OR). A biexponential transformation was applied to improve data visualization; 19106 SVF cells were stained per sample.

VII.2.5 Histopathologic examination and immunohistochemistry

Livers were fixed in buffered formalin and embedded in paraffin for hematoxylin-eosin (HE) and periodic-acid Schiff (PAS) histopathologic analysis as previously described [39,40].

VII.2.6 Statistical analysis

Statistical analysis was carried out with GraphPad™ Prism™ 7 (CA, USA). The normality of the data obtained was evaluated using Kolmogorov–Smirnov test. Accordingly, Kruskal–Wallis and Sidak's multiple comparison tests were applied and data depicted in median of all independent experiments. Differences among groups were considered significant when $P < 0.05$.

VII.3 Results and Discussion

Candidaemia has been increasing in the last decades, especially among individuals under chemotherapy programs as well as those HIV positive, hospitalized or catheterized [2,41]. *C. albicans* is still the most frequent isolated yeast, but *C. glabrata* has become one of the most threatening non-*Candida albicans Candida* (NCAC) spp., mostly due to high antifungal resistance [2,42]. Though human clinical data demonstrate that immunosuppression is a risk factor for *C. glabrata* infections, it is not an absolute prerequisite for *C. glabrata* candidiasis [43]. Therefore, increasing the knowledge on the host immune response to *C. glabrata* and studying the efficacy of chemotherapeutic approaches to treat infections caused by this fungus is thus of major value. The murine model is a suitable one to address both issues, alone or combined.

VII.3.1 Fungal Burden Progression Differs Substantially among Liver and Kidneys

The fungal burden of CD1 mice infected intravenously with *C. glabrata* biofilm cell suspensions and subsequently treated with echinocandins was assessed in the liver and kidneys 72 h post-infection. No difference was observed among the different infected groups.

In contrast to *C. albicans*, which can heavily infect the kidneys [44], it was clearly noticed a tropism of *C. glabrata* to the liver. High CFU counts were detected on this organ (Figure VII.1), contrasting to the low or non-detected CFU counts in the kidneys (data not shown). The low colonisation of this organ, as compared to liver or brain in immunocompetent mice systemically infected with *C. glabrata* was also reported by others [1,45–48]. Nevertheless, Kaur et al [47], Srikantha et al[48] and Brieland et al [46] confirmed that *C. glabrata* could be recovered after 7, 14 and 21 days, respectively in kidneys, liver, spleen, hearts, lungs, brains, and lungs. Atkinson et al [49] described that fungal burdens were of 10^4 to 10^8 in immunocompromised mice in spleen and kidneys. Nonetheless, is it important to state that the differences in mouse strains and immunocompetence status, *C. glabrata* strains, animal age and gender, or even the concentration of the inoculum used, do not allow a direct comparison of published data [45]. In addition, past *in vitro* reports have shown that susceptible *C. glabrata* strains can become resistant in less than four days of continuous culture with low doses of drugs, such as fluconazole [1,15] and echinocandins [50–53]. Thus, it is plausible that a fast increase of resistance could have been observed *in vivo*. Moreover, the inoculum exclusively contained biofilm cells, known to be even more resistant than the planktonic counterparts [54–60].

VII.3.2 Host immune response to hematogenously disseminated candidiasis

In contrast to the considerable work that has been described on the host immune response to *C. albicans*, the immune mechanisms elicited in the course of *C. glabrata* infections are far less explored. In *Candida* spp. infections, after the attachment, the ensuing step in pathogenesis is invasion, normally across the epithelial cell layer. However, *in vivo* invasion may also rely on endocytosis, instead of active penetration, with poor host cell damage and low pro-inflammatory response [45,61,62]. Although it is recognized that endothelial cells can internalize *C. albicans*, this was not observed for *C. glabrata*, which suggests that this endothelial cell activity may be species specific or restricted to *C. albicans* alone [63].

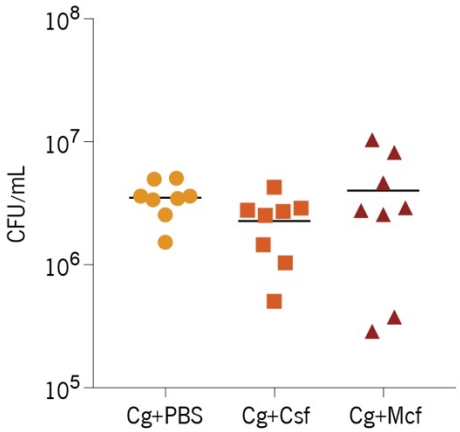

Figure VII.1. Liver fungal burden of CD1 mice 72 h after intravenously challenged with 1 × 10^8 biofilm cells plus two cycles of treatment with PBS, caspofungin (Csf) or micafungin (Mcf). Data are representative of two independent experiments. Each symbol represents an individual mouse, and horizontal bars are means of CFU numbers for each group. The obtained results are displayed as CFU/Liver. controls (naïve; PBS + Csf; PBS + Mcf) n = 2; Cg + Csf n = 8; Cg + Mcf = 8. No statistical differences were observed among infected groups (Kruskal–Wallis (Overall ANOVA $P < 0.05$) and post hoc Sidak's multiple comparison tests) (Cg - *Candida glabrata* ATCC2001).

Neutrophils and macrophages are in the first line of host immune defence against *Candida* spp. cells infecting the bloodstream or the endothelia [64–66]. Clinical observations and experimental studies have extensively demonstrated the major role of polymorphonuclear leukocytes in mediating host protection against systemic *C. albicans* infections [67–69]. Neutrophils have a Gr-1^{high} surface phenotype and macrophages typically express the F4/80 cell surface marker. Previous reports have shown that, in *C. albicans* infections, Gr-1^+ splenocytes may have immunosuppressive function and that F4/80^+ cells may have a pro-inflammatory role [70,71]. The expression of these two surface markers was analysed using flow cytometry in the spleen of CD1 mice 72 h upon infection i.v. with 1x10^8 *C. glabrata* biofilm cells. Myeloid cells (CD11b^+) displaying the following phenotypes F4/80^{high} Gr-1^{neg}, F4/80^{high} Gr-1^{high}, and F4/$80^{neg/low}$ Gr-1^{high} were respectively considered macrophages, inflammatory monocytes and neutrophils [72]. Used gating strategies are shown in Figure VII.2. As shown in Figure VII.3A, a significant increase in the numbers of inflammatory monocytes was observed in the spleen of infected mice, while those of neutrophils and macrophages remained within control values. No significant differences were however observed among treated groups.

These results are in accordance with previous reports [45,73,74]. In fact, contrarily to the marked neutrophil infiltration that is characteristically observed during *C. albicans* infections, *C. glabrata* either does not stimulate or is able to suppress neutrophil attraction and is more associated with mononuclear cells [45,73]. The existence of hyphae and occurrence of host cell damage in *C.*

albicans infections might account for a stronger pro-inflammatory response than in *C. glabrata* infections [74].

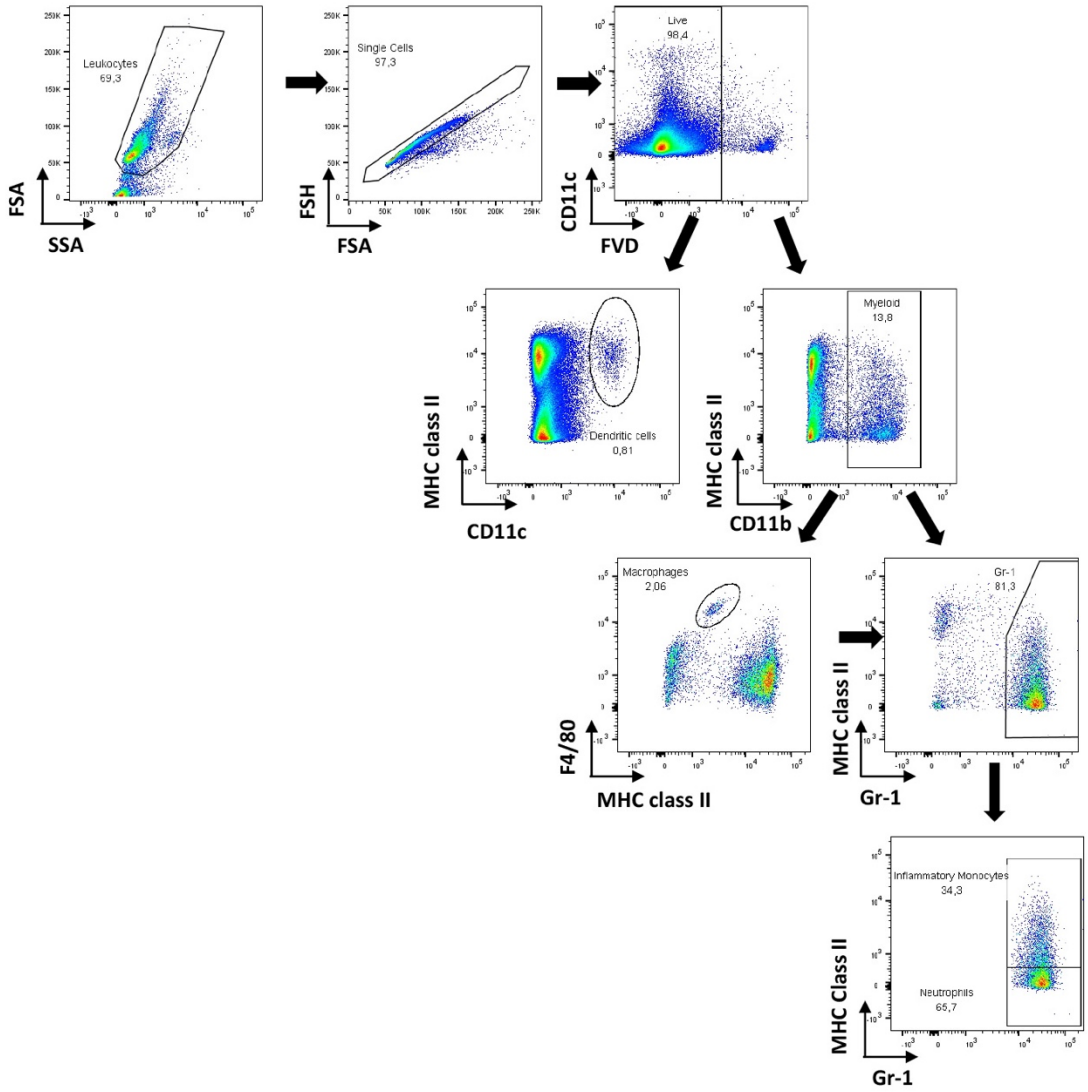

Figure VII.2 Gating strategy applied for the flow cytometry data analysis.

Additionally, other reports have shown that *C. glabrata* is recognized and phagocytized by macrophages at a much higher rate than *C. albicans* [75]. After recognizing pathogens, macrophages release cytokines that help coordinate the immune response. However, when *C. glabrata* is internalized by macrophages, it interferes with the phagosome maturation process [76], surviving through autophagy and replicating inside the phagosome until the eventual bursting of the phagocyte [47,76,77]. Here, no elevated numbers of macrophages were detected in the spleen of infected mice, compared to noninfected controls (Figure VIII.3C), which indicates that recruitment or local proliferation of these cells do not occur in response to *C. glabrata*.

In addition to macrophages, dendritic cells (DC) play a major role in the induction of the T cell-mediated immune response to *Candida* spp. infections [77,80] and may determine the infection outcome [81,82]. DC are able to modulate adaptive responses, depending on the *Candida* spp. morphotype encountered [64,65,83]. DC can initiate and shape the antimicrobial immunity and, since candidiasis appears frequently in immunocompromised patients, these cells may hold the key to new antifungal strategies [84]. Thus, the numbers of splenic conventional DC, defined as $CD11c^{high}$ cells, and surface maturation markers were assessed upon *C. glabrata* systemic infection (Figure VII.4). A slight increase in the numbers of splenic DC, compared to noninfected controls was observed in the infected mice, indicating that *C. glabrata* promoted the mobilization of these cells to the spleen or promoted their local proliferation. DC surface expression of the costimulatory molecule CD86, as evaluated by the mean fluorescence intensities (MFIs) due to antibody staining (Figure VII.4A and VII.4B) was elevated in the infected mice, showing that *C. glabrata* induced the maturation of these cells. However, the stimulatory effect was not different among treated and nontreated groups.

Contrastingly, expression of MHC class II molecules on the surface of splenic DC of mice infected with this yeast was found below control levels, an effect that reached statistical difference in mice treated with caspofungin. As CD86 expression in the infected mice was found elevated, it is unlikely that this could represent a suppressive mechanism and could just be subsequent to a previous stimulatory effect. A kinetic study would be necessary to elucidate this point.

The expression of CD80, CD86 and MHC class II molecules on the surface of inflammatory monocytes was observed to be similar or slightly lower in the infected groups, compared to non-infected controls (Figure VII.5A, VII.5B and VII.5C). Likewise, and as observed on DC, no differences were observed among infected mouse groups indicating that the treatment did not affect the expression of these activation markers on these innate immune cell populations. Finally, liver and kidney histopathology was analysed in the infected mice, as these organs are a preferred target in i.v. *Candida spp.* infections [45,86]. As could be expected, no yeasts were found in the non-challenged control groups, and their organs presented no significant histological alterations.

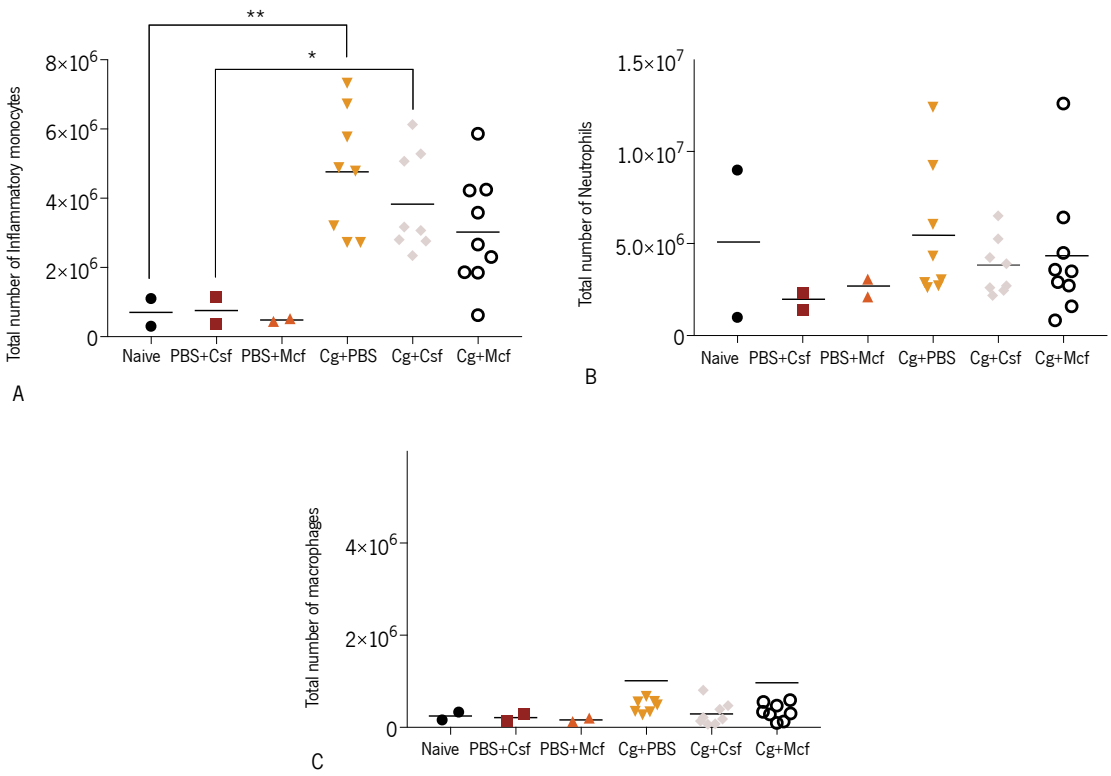

Figure VII.3. CD1 mice were challenged intravenously with 1 × 10^8 biofilm cells and then treated with PBS, caspofungin (Csf) or micafungin (Mcf). The obtained results are displayed as the total number of cells of indicated populations: (A) inflammatory monocytes; (B) neutrophils and (C) macrophages. The used number of animals was as follows: controls (naïve; PBS + Csf; PBS + Mcf) n = 2; Cg + Csf n = 8; Cg + Mcf = 8. Statistical differences among controls and infected groups were evaluated using One-way ANOVA, Kruskal–Wallis and post hoc Sidak's multiple comparison tests (Overall ANOVA P < 0.05) (Cg - *Candida glabrata* ATCC2001. ** P<0.05; ** P<0.001).*

Challenged mice showed inflammatory infiltrates in the liver, and less markedly in the kidneys (nontreated and treated groups). The presence of polymorphonuclear cells was observed, but in general, the infiltration remained mostly mononuclear. Yeasts were found in the liver, but not in the kidneys of treated and nontreated challenged groups. This fact corroborated the low CFU counts found in the kidneys.

Together, these observations confirmed *C. glabrata* as a low inflammatory species and indicated that two-dose treatment with caspofungin and micafungin do not have a significant impact on liver and kidney fungal burden, or recruited inflammatory infiltrate, when mice are i.v. infected with *C. glabrata* biofilm-grown cells.

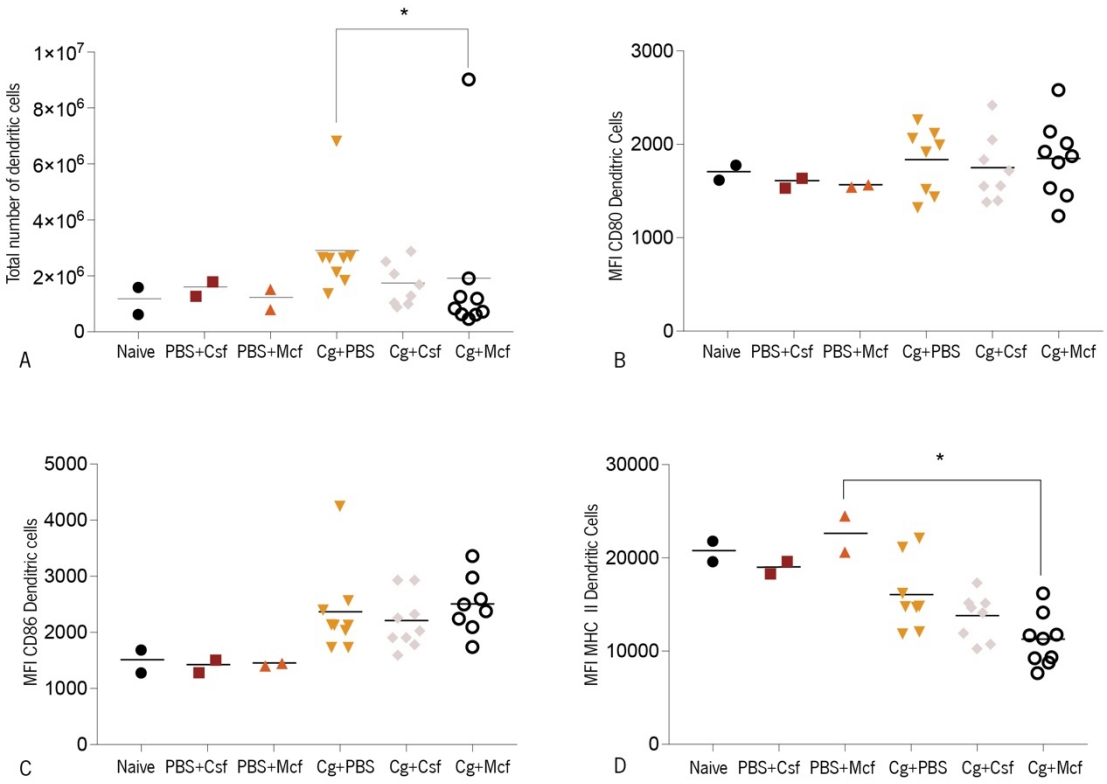

Figure VII.4. CD1 mice were challenged intravenously with 1 × 10^8 biofilm cells and then treated with PBS, caspofungin (Csf) or micafungin (Mcf). The obtained results are displayed as the mean fluorescence intensities (MFI) due to antibody staining against (A) CD80, (B) CD86 and (C) MHC class II on the surface of dendritic cells. The following number of animals were used: controls (naïve; PBS + Csf; PBS + Mcf) n = 2; Cg + Csf n = 8; Cg + Mcf = 8. Statistical differences among infected groups were evaluated using Kruskal–Wallis (Overall ANOVA $P < 0.05$) and post hoc Sidak's and Dunn's multiple comparisons tests (* $P>0.05$) (Cg - *Candida glabrata* ATCC2001).

Finally, liver and kidney histopathology was analysed in the infected mice, as these organs are a preferred target in i.v. *Candida* spp. infections [45,86]. As could be expected, no yeasts were found in the non-challenged control groups, and their organs presented no significant histological alterations (Figure VII.6). Challenged mice showed inflammatory infiltrates in the liver, and less markedly in the kidneys (nontreated and treated groups, data not shown). The presence of polymorphonuclear cells was observed, but in general, the infiltration remained mostly mononuclear. Yeasts were found in the liver (Figure VII.6), but not in the kidneys (data not shown) of treated and nontreated challenged groups. This fact corroborated the low CFU counts found in the kidneys.

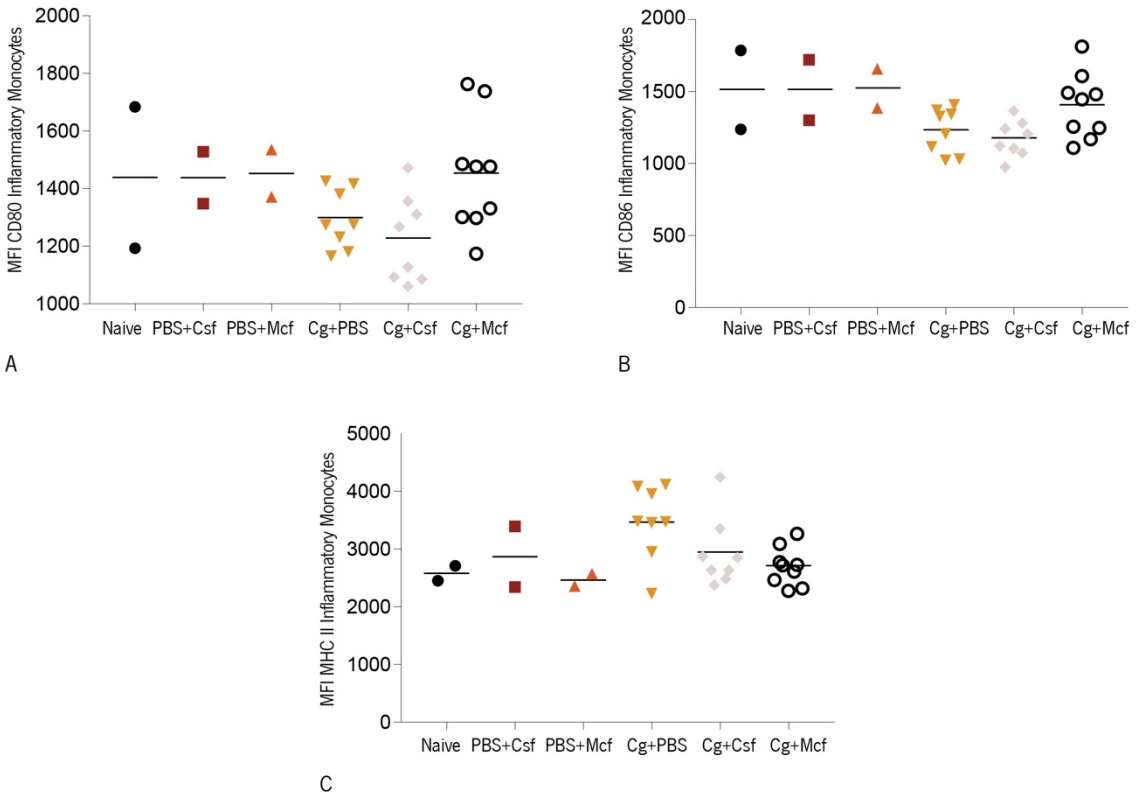

Figure VII.5. CD1 mice were challenged intravenously with 1 × 10^8 biofilm cells and then treated with PBS, caspofungin (Csf) or micafungin (Mcf). The obtained results are displayed as mean fluorescence intensities (MFI) in CD80 (A), in CD86 (B) and in MHC II (C) on inflammatory monocytes. Number of animals per group: controls (naïve; PBS + Csf; PBS + Mcf) n = 2; Cg + Csf n = 8; Cg + Mcf = 8. Statistical differences among infected groups were evaluated using Kruskal–Wallis (Overall ANOVA $P < 0.05$) and post hoc Sidak's multiple comparison tests (Cg - *Candida glabrata* ATCC2001).

Together, these observations confirmed *C. glabrata* as a low inflammatory species and indicated that two-dose treatment with caspofungin and micafungin do not have a significant impact on liver and kidney fungal burden, or recruited inflammatory infiltrate, when mice are i.v. infected with *C. glabrata* biofilm-grown cells.

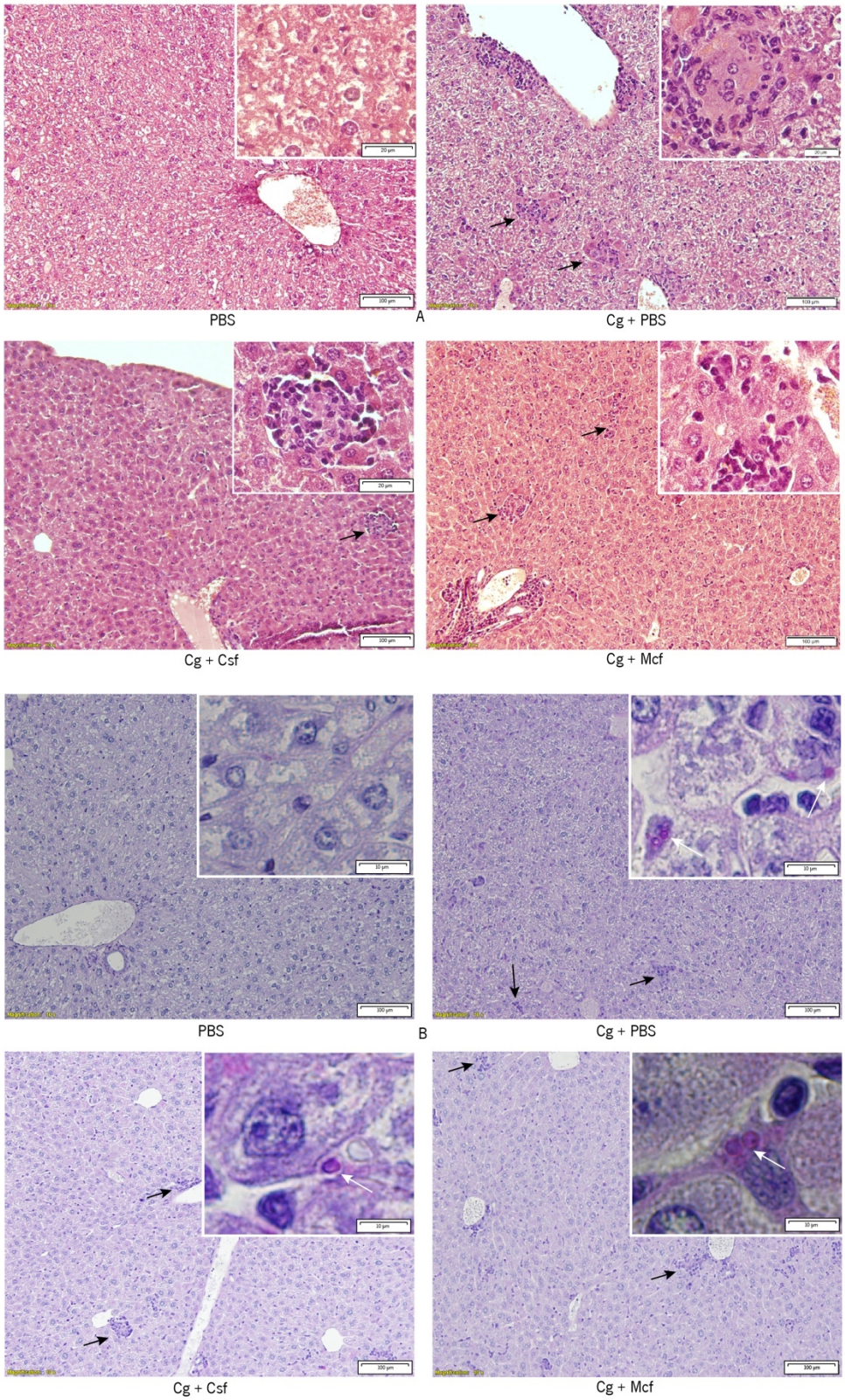

Figure VII.6. Analysis of liver histology in CD1 mice. (A) Representative hematoxylin-eosin and (B) Periodic-Schiff (PAS)-stained examples of liver tissue from the indicated mouse groups. Black arrows denote inflammatory infiltrates that were mostly of mononuclear type. Insets correspond to higher magnification micrographs. White arrows indicate PAS-stained *Candida glabrata* ATCC2001 cells. Scale bars are shown and apply to similar sized micrographs or insets. Bars: 100, 20 or 10 µm, as indicated.

References

1. Brunke, S.; Seider, K.; Fischer, D.; Jacobsen, I. D.; Kasper, L.; Jablonowski, N.; Wartenberg, A.; Bader, O.; Enache-Angoulvant, A.; Schaller, M.; D 'enfert, C.; Hube, B.; Brunke, S.; Seider, K.; Fischer, D.; Id, J.; Kasper, L. One Small Step for a Yeast -Microevolution within Macrophages Renders Candida glabrata Hypervirulent Due to a Single Point Mutation. *PLoS Pathog.* **2014**, *10*, e1004478.
2. Rodrigues, C.; Rodrigues, M.; Silva, S.; Henriques, M. Candida glabrata Biofilms: How Far Have We Come? *J. Fungi* **2017**, *3*, 11.
3. Archimedes, D.; Carolina, A.; Souza, R.; Colombo, A. L. Revisiting Species Distribution and Antifungal Susceptibility of Candida Bloodstream Isolates from Latin American Medical Centers. *J. Fungi* **2017**, *3*, 24.
4. Pfaller, M. A.; Diekema, D. J. Epidemiology of invasive candidiasis: A persistent public health problem. *Clin. Microbiol. Rev.* **2007**, *20*, 133–163.
5. Fidel, P.; Vazquez, J.; Sobel, J. Candida glabrata: review of epidemiology, pathogenesis, and clinical disease with comparison to C. albicans. *Clin. Microbiol. Rev.* **1999**, *12*, 80–96.
6. Arendrup, M. C. Candida and candidaemia. Susceptibility and epidemiology. *Dan. Med. J.* **2013**, *60*, B4698.
7. Shin, J. H.; Chae, M. J.; Song, J. W.; Jung, S.-I.; Cho, D.; Kee, S. J.; Kim, S. H.; Shin, M. G.; Suh, S. P.; Ryang, D. W. Changes in karyotype and azole susceptibility of sequential bloodstream isolates from patients with Candida glabrata candidemia. *J. Clin. Microbiol.* **2007**, *45*, 2385–91.
8. Bader, O.; Schwarz, A.; Kraneveld, E. A.; Tangwattanchuleeporn, M.; Schmidt, P.; Jacobsen, M. D.; Gross, U.; De Groot, P. W. J.; Weig, M. Gross Karyotypic and Phenotypic Alterations among Different Progenies of the Candida glabrata CBS138/ ATCC2001 Reference Strain. *PLoS One* **2012**, *7*, e52218.
9. Poláková, S.; Blume, C.; Zárate, J. A.; Mentel, M.; Jørck-Ramberg, D.; Stenderup, J.; Piskur, J. Formation of new chromosomes as a virulence mechanism in yeast Candida glabrata. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 2688–93.
10. Hachem, R.; Hanna, H.; Kontoyiannis, D.; Jiang, Y.; Raad, I. The changing epidemiology of invasive candidiasis: Candida glabrata and Candida krusei as the leading causes of candidemia in hematologic malignancy. *Cancer* **2008**, *112*, 2493–9.
11. Malani, A.; Hmoud, J.; Chiu, L.; Carver, P. L.; Bielaczyc, A.; Kauffman, C. A. Candida glabrata Fungemia: Experience in a Tertiary Care Center. *Clin. Infect. Dis.* **2005**, *41*, 975–981.
12. Playford, E. G.; Marriott, D.; Nguyen, Q.; Chen, S.; Ellis, D.; Slavin, M.; Sorrell, T. C. Candidemia in nonneutropenic critically ill patients: Risk factors for non-albicans Candida spp. *Crit. Care Med.* **2008**, *36*, 2034–2039.
13. Marriott, D. J.; Playford, E. G.; Chen, S.; Slavin, M.; Nguyen, Q.; Ellis, D.; Sorrell, T. C. Determinants of mortality in non-neutropenic ICU patients with candidaemia for the Australian Candidaemia Study. *Crit. Care* **2009**, *13*, 1–8.
14. Sipsas, N. V.; Lewis, R. E.; Tarrand, J.; Hachem, R.; Rolston, K. V.; Raad, I. I.; Kontoyiannis, D. P. Candidemia in patients with hematologic malignancies in the era of new antifungal agents (2001-2007). *Cancer* **2009**, *115*, 4745–4752.
15. Borst, A.; Raimer, M. T.; Warnock, D. W.; Morrison, C. J.; Arthington-Skaggs, B. A. Rapid Acquisition of Stable Azole Resistance by Candida glabrata Isolates Obtained before the Clinical Introduction of Fluconazole. *Antimicrob. Agents Chemother.* **2005**, *49*, 783–787.
16. Sanglard, D.; Ischer, F.; Calabrese, D.; Majcherczyk, P. A.; Bille, J. The ATP binding cassette transporter gene CgCDR1 from Candida glabrata is involved in the resistance of clinical isolates to azole antifungal agents. *Antimicrob. Agents Chemother.* **1999**, *43*, 2753–65.
17. McCarty, T. P.; Pappas, P. G. Invasive Candidiasis. *Infect. Dis. Clin. North Am.* 2016, *30*.
18. Lockhart, S. R.; Iqbal, N.; Cleveland, A. A.; Farley, M. M.; Harrison, L. H.; Bolden, C. B.; Baughman, W.; Stein, B.; Hollick, R.; Park, B. J.; Chiller, T. Species Identification and Antifungal Susceptibility Testing of Candida Bloodstream Isolates from Population-Based Surveillance Studies in Two U.S. Cities from 2008 to 2011. *J. Clin. Microbiol.* **2012**, *50*, 3435–3442.
19. Pfaller, □ M; Boyken, L.; Hollis, R.; Kroeger, J.; Messer, S.; Tendolkar, S.; Diekema, D. Use of Epidemiological Cutoff Values To Examine 9-Year Trends in Susceptibility of Candida Species to Anidulafungin, Caspofungin, and Micafungin. *J. Clin. Microbiol.* **2011**, *49*, 624–629.
20. Cleary, J. D.; Garcia-Effron, G.; Chapman, S. W.; Perlin, D. S. Reduced Candida glabrata Susceptibility secondary to an FKS1 Mutation Developed during Candidemia Treatment. *Antimicrob. Agents Chemother.* **2008**, *52*, 2263–2265.
21. Thompson, G. R.; Wiederhold, N. P.; Vallor, A. C.; Villareal, N. C.; Lewis, J. S.; Patterson, T. F. Development of caspofungin resistance following prolonged therapy for invasive candidiasis secondary to Candida glabrata infection. *Antimicrob. Agents Chemother.* **2008**, *52*, 3783–5.
22. Chapeland-Leclerc, F.; Hennequin, C.; Papon, N.; Noël, T.; Girard, A.; Socié, G.; Ribaud, P.; Lacroix, C. Acquisition of Flucytosine, Azole, and Caspofungin Resistance in Candida glabrata Bloodstream Isolates Serially Obtained from a

Hematopoietic Stem Cell Transplant Recipient. *Antimicrob. Agents Chemother.* **2010**, *54*, 1360–1362.
23. Durán-Valle, M. T.; Gago, S.; Gómez-López, A.; Cuenca-Estrella, M.; Jiménez Díez-Canseco, L.; Gómez-Garcés, J. L.; Zaragoza, O. Recurrent Episodes of Candidemia Due to Candida glabrata with a Mutation in Hot Spot 1 of the FKS2 Gene Developed after Prolonged Therapy with Caspofungin. *Antim Agents Chemoter.* **2012**, *56*, 3417–3419.
24. Shields, R. K.; Nguyen, M. H.; Press, E. G.; Kwa, A. L.; Cheng, S.; Du, C.; Clancy, C. J. The presence of an FKS mutation rather than MIC is an independent risk factor for failure of echinocandin therapy among patients with invasive candidiasis due to Candida glabrata. *Antimicrob. Agents Chemother.* **2012**, *56*, 4862–9.
25. Pfeiffer, C. D.; Garcia-Effron, G.; Zaas, A. K.; Perfect, J. R.; Perlin, D. S.; Alexander, B. D. Breakthrough Invasive Candidiasis in Patients on Micafungin. *J. Clin. Microbiol.* **2010**, *48*, 2373–2380.
26. Bizerra, F. C.; Jimenez-Ortigosa, C.; Souza, A. C. R.; Breda, G. L.; Queiroz-Telles, F.; Perlin, D. S.; Colombo, A. L. Breakthrough candidemia due to multidrug-resistant Candida glabrata during prophylaxis with a low dose of micafungin. **2014**, *58*, 2438–40.
27. Pham, C. D.; Iqbal, N.; Bolden, C. B.; Kuykendall, R. J.; Harrison, L. H.; Farley, M. M.; Schaffner, W.; Beldavs, Z. G.; Chiller, T. M.; Park, B. J.; Cleveland, A. a.; Lockhart, S. R. Role of FKS mutations in Candida glabrata: MIC values, echinocandin resistance, and multidrug resistance. *Antimicrob. Agents Chemother.* **2014**, *58*, 4690–4696.
28. Park, S.; Kelly, R.; Kahn, J. N. N.; Robles, J.; Hsu, M. J. M.-J.; Register, E.; Li, W.; Vyas, V.; Fan, H.; Abruzzo, G.; Flattery, A.; Gill, C.; Chrebet, G.; Parent, S. A. A.; Kurtz, M.; Teppler, H.; Douglas, C. M. M.; Perlin, D. S. S. Specific substitutions in the echinocandin target Fks1p account for reduced susceptibility of rare laboratory and clinical Candida sp. isolates. *Antimicrob. Agents Chemother.* **2005**, *49*, 3264–3273.
29. Perlin, D. S.; Teppler, H.; Donowitz, G. R.; Maertens, J. A.; Baden, L. R.; al., et; Milne, S.; Brown, A. J.; Gow, N. A. Resistance to echinocandin-class antifungal drugs. *Drug Resist. Updat.* **2007**, *10*, 121–30.
30. Garcia-Effron, G.; Chua, D. J.; Tomada, J. R.; Dipersio, J.; Perlin, D. S.; Ghannoum, M.; Bonilla, H. Novel FKS Mutations Associated with Echinocandin Resistance in Candida Species. *Antimicrob. Agents Chemother.* **2010**, *54*, 2225–2227.
31. Coenye, T.; Bjarnsholt, T. The complexity of microbial biofilm research – an introduction to the 3 rd Thematic Issue on Biofilms. *Pathog. Dis.* **2016**.
32. Zarnowski, R.; Westler, W. M.; Lacmbouh, G. A.; Marita, J. M.; Bothe, J. R.; Bernhardt, J.; Sahraoui, A. L. H.; Fontainei, J.; Sanchez, H.; Hatfeld, R. D.; Ntambi, J. M.; Nett, J. E.; Mitchell, A. P.; Andes, D. R. Novel entries in a fungal biofilm matrix encyclopedia. *MBio* **2014**, *5*, 1–13.
33. LaFleur, M. D.; Kumamoto, C. A.; Lewis, K. Candida albicans biofilms produce antifungal-tolerant persister cells. *Antimicrob. Agents Chemother.* **2006**, *50*, 3839–3846.
34. Fonseca, E.; Silva, S.; Rodrigues, C. F.; Alves, C. T.; Azeredo, J.; Henriques, M. Effects of fluconazole on Candida glabrata biofilms and its relationship with ABC transporter gene expression. *Biofouling* **2014**, *30*, 447–57.
35. Rodrigues, C. F.; Gonçalves, B.; Rodrigues, M. E.; Silva, S.; Azeredo, J.; Henriques, M. The Effectiveness of Voriconazole in Therapy of Candida glabrata's Biofilms Oral Infections and Its Influence on the Matrix Composition and Gene Expression. *Mycopathologia* **2017**, *182*, 653–664.
36. Rodrigues, C.; Henriques, M. Oral mucositis caused by Candida glabrata biofilms: failure of the concomitant use of fluconazole and ascorbic acid. *Ther. Adv. Infect. Dis.* **2017**, *1*.
37. Arendrup, M.; Perlin, D.; Jensen, R.; Howard, S.; Goodwin, J.; Hopec, W. Differential in vivo activities of anidulafungin, caspofungin, and micafungin against Candida glabrata isolates with and without FSK resistance mutations. *Antim Agents Chemoter.* **2012**, 2435–2442.
38. Andes, D.; Diekema, D. J.; Pfaller, M. A.; Bohrmuller, J.; Marchillo, K.; Lepak, A.; Andes, □ D; Diekema, D. J.; Pfaller, M. A.; Bohrmuller, J.; Marchillo, K.; Lepak, A. In Vivo comparison of the pharmacodynamic targets for echinocandin drugs against Candida species. *Antimicrob. Agents Chemother.* **2010**, *54*, 2497–2506.
39. Teixeira, L.; Moreira, J.; Melo, J.; Bezerra, F.; Marques, R. M.; Fer-Reirinha, P.; Correia, A.; Monteiro, M. P.; Ferreira, P. G.; Vilanova, M. Immune response in the adipose tissue of lean mice infected with the protozoan parasite Neospora caninum. *Immunology* **2014**, *145*, 242–257.
40. Kumar, R.; Saraswat, D.; Tati, S.; Edgerton, M. Novel Aggregation Properties of Candida albicans Secreted Aspartyl Proteinase Sap6 Mediate Virulence in Oral Candidiasis. *Infect. Immun.* **2015**, *83*, 2614–26.
41. Lockhart, S. R. Current Epidemiology of Candida Infection. *Clin. Microbiol. Newsl.* **2014**, *36*.
42. Silva, S.; Rodrigues, C.; Araújo, D.; Rodrigues, M.; Henriques, M. Candida Species Biofilms' Antifungal Resistance. *J. Fungi* **2017**, *3*, 8.
43. Safdar, A.; Bannister, T. W.; Safdar, Z.; Ellis, M.; Ain, A. The predictors of outcome in immunocompetent patients with hematogenous candidasis. *Int. J. Infect. Dis.* **2004**, *8*, 180–186.
44. Correia, A.; Lermann, U.; Teixeira, L.; Cerca, F.; Botelho, S.; Gil Da Costa, R. M.; Sampaio, P.; Gärtner, F.; Morschhäuser, J.; Vilanova, M.; Pais, C. Limited Role of Secreted Aspartyl Proteinases Sap1 to Sap6 in Candida albicans Virulence and Host Immune Response in Murine Hematogenously Disseminated Candidiasis. *Infect. Immun.* **2010**, *78*, 4839–4849.

45. Jacobsen, I. D.; Brunke, S.; Seider, K.; Schwarzmüller, T.; Firon, A.; D 'enfért, C.; Kuchler, K.; Hube, B. Candida glabrata Persistence in Mice Does Not Depend on Host Immunosuppression and Is Unaffected by Fungal Amino Acid Auxotrophy. *Infect. Immun.* **2010**, *78*, 1066–1077.
46. Brieland, J.; Essig, D.; Jackson, C.; Frank, D.; Loebenberg, D.; Menzel, F.; Arnold, B.; DiDomenico, B.; Hare, R. Comparison of pathogenesis and host immune responses to Candida glabrata and Candida albicans in systemically infected immunocompetent mice. *Infect. Immun.* **2001**, *69*, 5046–55.
47. Kaur, R.; Ma, B.; Cormack, B. P. A family of glycosylphosphatidylinositol-linked aspartyl proteases is required for virulence of Candida glabrata. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 7628–33.
48. Srikantha, T.; Daniels, K. J.; Wu, W.; Lockhart, S. R.; Yi, S.; Sahni, N.; Ma, N.; Soll Correspondence, D. R.; Soll, D. R. Dark brown is the more virulent of the switch phenotypes of Candida glabrata. *Microbiology* **2008**, *154*, 3309–3318.
49. Atkinson, B. A.; Bouthet, C.; Bocanegra, R.; Correa, A.; Luther, M. F.; Graybill, J. R. Comparison of fluconazole, amphotericin B and flucytosine in treatment of a murine model of disseminated infection with Candida glabrata in immunocompromised mice. *J. Antimicrob. Chemother.* **1995**, *35*, 631–40.
50. Pfaller, M. A.; Moet, G. J.; Messer, S. A.; Jones, R. N.; Castanheira, M. Candida bloodstream infections: comparison of species distributions and antifungal resistance patterns in community-onset and nosocomial isolates in the SENTRY Antimicrobial Surveillance Program, 2008-2009. *Antimicrob. Agents Chemother.* **2011**, *55*, 561–6.
51. Healey, K. R.; Ortigosa, C. J.; Shor, E.; Perlin, D. S. Genetic Drivers of Multidrug Resistance in Candida glabrata. *Front. Microbiol.* **2016**, *7*, 1–9.
52. Morio, F.; Jensen, R. H.; Pape, P. Le; Arendrup, M. C. Molecular basis of antifungal drug resistance in yeasts. *Int. J. Antimicrob. Agents* **2017**, *17*.
53. Perlin, D. S. Mechanisms of echinocandin antifungal drug resistance. *Ann N Y Acad Sci* **2015**, 1–11.
54. Ferrari, S.; Sanguinetti, M.; De Bernardis, F.; Torelli, R.; Posteraro, B.; Vandeputte, P.; Sanglard, D. Loss of mitochondrial functions associated with azole resistance in Candida glabrata results in enhanced virulence in mice. *Antimicrob. Agents Chemother.* **2011**, *55*, 1852–60.
55. Al-fattani, M. a; Douglas, L. J. Penetration of Candida Biofilms by Antifungal Agents. *Antimicrob Agents Chemother* **2004**, *48*, 3291–3297.
56. De Luca, C.; Guglielminetti, M.; Ferrario, A.; Calabrò, M.; Casari, E. Candidemia: species involved, virulence factors and antimycotic susceptibility. *New Microbiol.* **2012**, *35*, 459–468.
57. Grandesso, S.; Sapino, B.; Mazzuccato, S.; Solinas, M.; Bedin, M.; D'Angelo, M.; Gion, M. Study on in vitro susceptibility of Candida spp. isolated from blood culture. *Infect Med.* **2012**, *20*, 25–30.
58. Lewis, R.; Kontoyiannis, D.; Darouiche, R.; Raad, I.; Prince, R. Antifungal activity of amphotericin B, fluconazole, and voriconazole in an in vitro model of Candida catheter-related bloodstream infection. *Antimicrob Agents Chemother.* **2002**, *46*, 3499–505.
59. Donlan, R.; Costerton, J. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.* **2002**, *15*, 167–193.
60. Basso, L. R.; Gast, C. E.; Mao, Y.; Wong, B. Fluconazole Transport into Candida albicans Secretory Vesicles by the Membrane Proteins Cdr1p, Cdr2p, and Mdr1p. *Eukaryot Cell* **2010**, *9*, 960–970.
61. Schaller, M.; Sander, C. A.; Korting, H. C.; Mailhammer, R.; Grassl, G.; Hube, B. Infection of Human Oral Epithelia with Candida Species Induces Cytokine Expression Correlated to the Degree of Virulence. *J. Invest. Dermatol.* **2002**, *118*, 652–657.
62. Li, L.; Kashleva, H.; Dongari-Bagtzoglou, A. Cytotoxic and cytokine inducing properties of Candida glabrata in single and mixed oral infection models.
63. Filler, S. G.; Swerdloff, J. N.; Hobbs, C.; Luckett, P. M. Penetration and Damage of Endothelial Cells by Candida albicans. *Infect. Immun.* **1995**, *63*, 976–983.
64. Netea, M. G.; Joosten, L. A.; van der Meer, J. W.; Kullberg, B. J.; van de Veerdonk, F. L. Immune defence against Candida fungal infections. *Nat Rev Immunol* **2015**, *15*.
65. Shoham, S.; Levitz, S. M. The immune response to fungal infections. *Br. J. Haematol.* **2005**, *129*, 569–582.
66. V Zquez-torres, A. S.; Balish, E. Macrophages in Resistance to Candidiasis. *Microbiol. Mol. Biol. Rev.* **1997**, *61*, 170–192.
67. Ehrensaft, D. V; Epstein, R. B.; Sarpel, S.; Andersen, B. R. Disseminated candidiasis in leukopenic dogs. *Proc. Soc. Exp. Biol. Med.* **1979**, *160*, 6–10.
68. Elin, R. J.; Edelin, J. B.; Wolff, S. M. Infection and Immunoglobulin Concentrations in Chediak-Higashi Mice. *Infect. Immun.* **1974**, *10*, 88–91.
69. Holm, H. W.; Marwin, R. M. Effects of surface active agents on the susceptibility of Swiss mice to Candida albicans. *Mycopathol. Mycol. Appl.* **1967**, *33*, 186–92.
70. Sharpe, H.; Romani Arlene Cenci, L.; Pitzurra, L.; Spreca, A.; Kopf, M.; Mencacci, A.; Montagnoli, C.; Bacci, A.; Cenci, E.; Sharpe, A. H.; Romani, L. CD80+ Gr-1+ Myeloid Cells Inhibit Development of Antifungal Th1 Immunity in

Mice with Candidiasis. *J Immunol Ref.* **2017**, *169*, 3180–3190.
71. Taylor, P. R.; Tsoni, S. V.; Willment, J. A.; Dennehy, K. M.; Rosas, M.; Findon, H.; Haynes, K.; Steele, C.; Botto, M.; Gordon, S.; Brown, G. D. Dectin-1 is required for β-glucan recognition and control of fungal infection. *Nat Immunol.* **2007**, *8*, 31–38.
72. Taylor, P. R.; Brown, G. D.; Geldhof, A. B.; Martinez-Pomares, L.; Gordon, S. Pattern recognition receptors and differentiation antigens define murine myeloid cell heterogeneity ex vivo. *Eur. J. Immunol.* **2003**, *33*, 2090–2097.
73. Westwater, C.; Schofield, D. A.; Nicholas, P. J.; Paulling, E. E.; Balish, E. *Candida glabrata* and *Candida albicans* ; dissimilar tissue tropism and infectivity in a gnotobiotic model of mucosal candidiasis. *FEMS Immunol. Med. Microbiol.* **2007**, *51*, 134–139.
74. Brunke, S.; Hube, B. Two unlike cousins: Candida albicans and C. glabrata infection strategies. *Cell. Microbiol.* **2013**, *15*, 701–8.
75. Keppler-Ross, S.; Douglas, L.; Konopka, J. B.; Dean, N. Recognition of Yeast by Murine Macrophages Requires Mannan but Not Glucan. *Eukaryot. Cell* **2010**, *9*, 1776–1787.
76. Seider, K.; Brunke, S.; Schild, L.; Jablonowski, N.; Wilson, D.; Majer, O.; Dagmar, B.; Haas, A.; Kuchler, K.; Schaller, M.; Huber, B. The Facultative Intracellular Pathogen Candida glabrata Subverts Macrophage Cytokine Production and Phagolysosome Maturation. *J. Immunol.* **2011**, *187*, 3072–3086.
77. Roetzer, A.; Gratz, N.; Kovarik, P.; Schüller, C. Autophagy supports Candida glabrata survival during phagocytosis. *Cell. Microbiol.* **2010**, *12*, 199–216.
78. Romani, L.; Mencacci, A.; Cenci, E.; Del Sero, G.; Bistoni, F.; Puccetti, P. An immunoregulatory role for neutrophils in CD4+ T helper subset selection in mice with candidiasis. *J. Immunol.* **1997**, *158*, 2356–62.
79. Lionakis Jean K Lim Chyi-Chia Richard Lee Philip M Murphy, M. S. Organ-Specific Innate Immune Responses in a Mouse Model of Invasive Candidiasis. *J Innate Immun* **2011**, *3*, 180–199.
80. Jandric, Z.; Schuller, C. Stress response in Candida glabrata: pieces of a fragmented picture. *Futur. Mirobiology* **2011**, *6*, 1475–1484.
81. Bonifazi, P.; Zelante, T.; D'Angelo, C.; De Luca, A.; Moretti, S.; Bozza, S.; Perruccio, K.; Iannitti, R. G.; Giovannini, G.; Volpi, C.; Fallarino, F.; Puccetti, P.; Romani, L. Balancing inflammation and tolerance in vivo through dendritic cells by the commensal Candida albicans. *Mucosal Immunol.* **2009**, *2*, 362–374.
82. Moraes Nicola, A.; Casadevall, A.; Goldman, D. L. Fungal killing by mammalian phagocytic cells. *Curr Opin Microbiol* **2008**, *11*, 313–317.
83. Romani, L.; Montagnoli, C.; Bozza, S.; Perruccio, K.; Spreca, A.; Allavena, P.; Verbeek, S.; Calderone, R. A.; Bistoni, F.; Puccetti, P. The exploitation of distinct recognition receptors in dendritic cells determines the full range of host immune relationships with Candida albicans. *Int. Immunol.* **2004**, *16*, 149–61.
84. Shi, D.; Li, D.; Qingxin, Y.; Qiu, Y.; Yan, H.; Shen, Y.; Guixia, L.; Liu, W. Silenced suppressor of cytokine signaling 1 (SOCS1) enhances the maturation and antifungal immunity of dendritic cells in response to Candida albicans in vitro. *Immunol Res* **2015**, *61*, 206–218.
85. Wagner, R. D.; Vazquez-Torres, A.; Jones-Carson, J.; Warner, T.; Balish, E. B Cell Knockout Mice Are Resistant to Mucosal and Systemic Candidiasis of Endogenous Origin but Susceptible to Experimental Systemic Candidiasis. *J. Infect. Dis.* **1996**, *174*.
86. Ohno, N.; Uchiyama, M.; Tsuzuki, A.; Tokunaka, K.; Miura, N. N.; Adachi, Y.; Aizawa, M. W.; Tamura, H.; Tanaka, S.; Yadomae, T. Solubilization of yeast cell-wall β-(1→3)-D-glucan by sodium hypochlorite oxidation and dimethyl sulfoxide extraction. *Carbohydr. Res.* **1999**, *316*, 161–172.

VIII

Chapter VIII

General Discussion and Final Remarks

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General Discussion and Final Remarks

Candidiasis is a growing problem in immunosuppressed, hospitalized or catheterized patients. Despite of *C. albicans* continuing to be the main *Candida* spp. responsible for this disease, NCAC spp. have been increasing their responsibility in this matter, specially *C. glabrata*. This yeast has the important features of having an innate resistance to azoles and to rapidly develop resistance to other antifungal classes, being very difficult to treat, particularly when in biofilm form.

In addition to the known efflux-pumps, *C. glabrata* biofilms matrices have a biopolymeric net that protects the biofilm cells from the attack of any xenobiotic, as demonstrated in this work. Fluconazole was confirmed to stay in the matrices and in supernatants of the biofilms depending on the susceptibility and the resistance mechanisms of the *C. glabrata* biofilm cells. When compared to fluconazole, voriconazole showed to have a higher capacity to reach and eliminate the cells. This difference might be due to the fact that voriconazole is a new triazole, less used than fluconazole, and, therefore, with fewer resistance mechanisms associated. In fact, voriconazole showed to have a greater effect on *C. glabrata* biofilms, than fluconazole, highly reducing the biomass and entering the cells in upper concentration (a minimum of 43.37% of voriconazole vs a maximum of 5.56% of fluconazole). *ERG3*, *ERG6* and *ERG11* genes expression showed a general overexpression of these genes in the presence of both azole drugs, which displayed the high ability of *C. glabrata* to rapidly adapt to stress. Another cell adaptation recurrent along this work was the increase of matrices' polysaccharides (general polysaccharides and β-1,3-glucans) and a decrease in matrices' proteins, after a contact with most of the antifungal drugs.

It is well known that β-1,3-glucans are part of the cell wall and the biofilm matrices of *Candida* spp.. Moreover, they are associated to drug resistance, since they difficult the drugs' diffusion through the biofilm matrices and, ultimately, not allowing the agent to get to the yeast cells. These biochemical alterations result in denser biofilm matrices, inflexible and more protective for the biofilm's cells environment. In order to try to degrade these β-1,3-glucans and weaken the *C. glabrata* biofilm matrices, ascorbic acid was used concomitantly with routine clinical doses of fluconazole. It was shown that the association efficiently stimulated the degradation of the biofilm network, but also stimulated the growth of the *C. glabrata* (10% to 15%, with ascorbic acid alone)

due to several glucose monomers released by the β-glucans hydrolysis, and that were assimilated by the yeasts. So, although being a common and very important additive to antibiotics, ascorbic acid should not be used with antifungal agents to treat *Candida* spp. infections.

A very important antifungal class and with low rate of associated resistance, is the polyenes. Yet, *C. glabrata* showed capacity to, even though in a lesser extent, develop complex biofilms in the presence of amphotericin B in therapeutic concentrations. The biofilms presented a thick extracellular matrix, with an increase on the concentration of polysaccharides, especially β-1,3-glucans. The work highlighted the importance of the regular change of the medical devices in the hospitalized patients, since the results demonstrated that, even under strong pharmacotherapy, the *C. glabrata* biofilm formation occurs and can be a source of systemic infections. In addition, the evaluation of clinical concentrations of two different pharmaceutical formulations of amphotericin B – liposomal and deoxycholate – resulted in similar beneficial effects in the planktonic cells (MIC and MFC), but better results in the biofilms (MBEC) of *Candida* spp., were obtained when using the liposomal formulation. The higher efficacy of the liposomal formulation on the biofilm forms was specially noticed in *C. albicans* (3.57%) and *C. parapsilosis* (2.38%) and less expressive in *C. glabrata* (≥9.52%), demonstrating, once more, its resistant character.

The search for alternative therapies, as the combination of antifungals from different classes is crucial. An association of the amphotericin B deoxycholate with a recent triazole – posaconazole - was evaluated on 24-h-formed biofilms of *C. glabrata*. With no surprise, *C. glabrata* demonstrated to have an enhanced susceptibility to the polyene when compared to the azole, in both planktonic and biofilm form (Pcz reduced 1 Log_{10} CFU/cm^2 with 200 mg/L and AmB reduced 1 Log_{10} CFU/cm^2 with 2 mg/L), but both drugs showed a good capacity in reducing the biomass (highest values: Pcz - 77.6%, AmB - 64.2%, both $P < 0.0001$). Nevertheless, the majority of the interactions were as found indifferent (as FICI ≤ 4) and it was concluded that the combination did not bring an advantage in the treatment of biofilms of *C. glabrata* ATCC2001. It is thought that this may be related to the mechanism of each antifungal, which could reduce the target of each other.

Echinocandins are, presently, the first-line agents, for the treatment of systemic candidiasis. The susceptibility of several *Candida* spp. and, particularly, seven *C. glabrata* biofilms' strains to caspofungin and micafungin and the evaluation of the effects on the biofilms' matrix composition were assessed. The MIC values were species/strain dependent, but, in general, all species/strains were susceptible to both agents. MFC and MBEC of micafungin were, in general, higher and *C. glabrata* strains demonstrated to have similar resistant profiles to *C. albicans* SC5314, *C. tropicalis*

ATCC750 and *C. parapsilosis* ATCC22019. After the echinocandin exposure, the biomass decreased very effectively in all species/strains. Yet, contrarily to micafungin, caspofungin revealed a higher biomass reduction capacity (minumun of 70% of the biofilm reduction). After drugs contact, the SEM images showed disrupted biofilm cells, with a concave aspect, resultant from the mechanism of action of the echinocandins (non-competitive inhibition of β-1,3-glucan synthesis). Contrarily to the other antifungal classes, normally (with some exceptions), β-1,3-glucans showed to statistically significantly decrease in the biofilm matrices of *C. glabrata*, and in the other *Candida* spp.. *Candida glabrata* and *C. parapsilosis* indicated to have the highest concentrations on the biofilm matrices, which could be a probable cause of the higher resistant profile, comparing to *C. albicans* and *C. tropicalis*. The variations in the matrices compositions could be responsible and determine the effectivity in the echinocandin biofilm cells' responses. An additional cause of inconsistency in the drug response can be the chemical differences between the two echinocandins. Modifications described as critical for the antifungal activity were implemented on micafungin and are a plausible justification for the variations in drug responses found in this work.

The biofilm formation is narrowly connected to the adjustment on the expression or to the mutation of genes, which may result in failure of the treatment. Mature biofilms of *C. glabrata* were studied in terms of the gene expression of *BGL2, XOG1, FKS1, FKS2, GAS2* (genes related β-1,3-glucans production), *KNH1, UGP1* (genes related to β-1,6-glucans production), *and MNN2* (gene related to mannans production), after contact with fluconazole, amphotericin B, caspofungin or micafungin, by RT-qPCR. *BGL2, XOG1, GAS2* genes confirmed to have the highest expressions percentages and micafungin revealed to induce a general overexpression of all genes. This work revealed that, although β-1,6-glucans and mannans are an important part of the cell and biofilm matrices, *C. glabrata* biofilm cells seem to make a stronger effort for the replacement of β-1,3-glucans. Again, it was demonstrated that these biopolymers seem to have a greater role in the resistance to the antifungal drugs.

In order to better understand the effect of the mannans on the resistant profile of biofilm cells of *C. glabrata*, the effect of a knock-out (KO) of the *MNN2* gene was considered in biofilms of this species, with and without drugs (fluconazole, amphotericin B, caspofungin and micafungin). Initially, no visible differences between the cell walls of the *C. glabrata* strains (reference strain, mutant and parent) were visualized in the CLSM. However, it was shown that the mutant had a great biomass reduction, after an antifungal stress. Curiously, the mutant strain also showed a reduction in the biofilm susceptibility, when compared to the reference strain. A significant decline

of mannans in the mutant cell walls and matrices was detected (20% in the mutant strain and 50-60% for the reference and parent in the controls and 10% and 20%, respectively, after antifungal contact, $P<0.0005$). The biofilm cell walls composition accused an increase of quantity of β-1,3-glucans in the *C. glabrata* Δ*mnn2,* when compared with the other two, probably compensating the detected lack of mannans. This increase of β-1,3-glucans could explain the reduction in the biofilm susceptibility.

The evaluation of the effectiveness of caspofungin (Csf) and micafungin (Mcf) in the treatment of a systemic infection of *C. glabrata* derived exclusively from from biofilm cells was also assessed. Accordingly, CD1 mice were infected with 48 h-biofilm cells of *C. glabrata* and then treated with Csf or Mcf. Results showed that the fungal burden was higher in the liver than in the kidneys and that none of the echinocandins was effective in eradicating completely *C. glabrata* biofilm cells. There was a prevalent mononuclear immune response in the spleen, which was also noticed in liver and kidneys of the infected mice, as observed by histopathology analysis.

In summary, the alterations on biofilm the matrices' composition, the mechanisms of resistance of the biofilm cells (e.g. genes expression adjustments, the continuous activation of the efflux-pumps), and the chemical modifications on the drugs can partially explain and determine the effectiveness response to these antifungals on the biofilms infections of *C. glabrata.*

IX

Chapter IX
Work Perspectives

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Work Perspectives

In all *Candida* spp., and specifically in *C. glabrata*, the resistance to antifungals agents, the main role of the biofilm cells and of the matrix components in the resistance mechanisms, is still a broad field with many paths to be determined.

This thesis included some less explored techniques, with the intention of characterizing and evaluating the matrix biopolymers and their possible relation to the low sensitivity of *C. glabrata* to antifungal drugs. Some interesting results were obtained, but have also raised some questions, which are expected to be assessed in future studies, as discussed in the next topics.

1. It was clear that *C. glabrata* biofilms radically change when environmentally pressured (e.g. by antifungal drugs). Generally, these biochemical changes in the matrices composition, involve the production of matrix compounds that dense the biofilm, contributing to a higher biofilm cell protection, to both chemical and physical aggressions. This reaction is stronger with azoles (in low and, in some cases, high drug concentrations), but also happens with other antifungals' classes, in low doses. Oppositely to an increase in the production of polysaccharides, the protein production is lowered in stressed cells of *C. glabrata*, turning the matrices mostly constituted by long polymers of sugars or glycoproteins, which are believed to have a greater impact on the resistance to antifungal agents', by delaying its diffusion through the matrices. It would be interesting to evaluate the chemical bond of these polymers to the different molecules that belong to azoles, polyenes or echinocandins and which conditions trigger these chemical reactions.
2. The liposomal formulation of amphotericin B showed to be clinically more appealing for the treatment of *C. glabrata* biofilms (including others *Candida* spp.). Evaluating the other formulations that can be used and to add clinical isolates also seems essential.
3. In this work, the concomitant use of polyenes and azoles (amphotericin B and posaconazole) was found to be, in general, antagonist, which might be directly related to the mechanisms of action of each drug that low the target of the other. Ultimately, other associations and concentrations could be tested for the treatment of *C. glabrata* biofilms: e.g. posaconazole + chorhexidin; voriconazole + chorhexidin; fluconazole + chorhexidin; chorhexidin + nystatin.

4. Several genes responsible for the production of matrix components were shown to be significant in the change of biofilm composition and the biofilm cells' susceptibility. Further evaluations, through the construction of disrupted mutants, on how each gene influences the *C. glabrata* biofilm cells' (e.g. tolerance, resistance, biofilm form capacity) would be essential to better define the significante of the gene.

5. This work also showed that the *C. glabrata* biofilm cells have very different cell membrane composition in terms of mannans and β-glucans (lower mannans and higher β-glucans amount). Allied to the already known greater quantity of chitin in the cell wall, this may be responsible to the high resistance to the antifungals. A more profound evaluation of the cell wall and membrane compositions, after the drug expositions would be interesting.

6. The *in vivo* infection model showed that the *C. glabrata* biofilm cells have a much lower susceptibility to echinocandins and that the immune response in this species is quite different than the one triggered by *C. albicans*. An *in vivo* evaluation of the role of the different *C. glabrata* matrix components in the immune response could be interesting to analyse. Also, the use of mutants of *C. glabrata* with less capacity to procuce cell wall and matrix components could be analysed in terms of the capacity to induce different immune and drug response.

7. The exploration of other active compounds should be performed. Since the antifungal drugs are so scarce, old (e.g. essential oils) or new (e.g. *in silico*) compounds need to keep being explored, developed and evaluated.

In addition to the suggestions here posed, there are many other developments to be made in the field *C. glabrata* biofilm resistance to antifungals, for example the optimization of the clinical protocols to the infections derived to biofilms and the development of new biomarkers and molecules. Every little improvement in these areas will have a positive impact on the clinical practice and the capacity of the treatment of these serious infections, but also on the biopharmaceutical industry, which will ultimately lead to an increased capacity to fulfill the market needs.