KU LEUVEN

ARENBERG DOCTORAL SCHOOL
Faculty of Science

Universidad del Rosario

Doctorado en Ciencias Biomédicas
Escuela de Medicina y Ciencias de la Salud
Facultad de Ciencias Naturales y Matemáticas

The role of the *FKS1* gene in nosocomial *Candida albicans* isolates' virulence and antifungal resistance

Giovanni Rodríguez Leguizamón

Supervisors:

Prof. Patrick Van Dijck

Prof. Manuel Alfonso Patarroyo

Prof. Arley Gómez

Dissertation presented in partial fulfillment of the requirements for the degree of PhD in Science

October 2015

Preface - Acknowledgements

This work describes and is aimed at a better understanding of the pattern of *Candida albicans* nosocomial infection by using molecular biology, conventional mycology and proteomics tools, exploring the role of the *FKS1* gene as a key factor regarding the fungus' cell wall stability which has implications for this significant microorganism's phenotypical pattern.

It is thus appropriate that I should acknowledge and express my heartfelt gratitude to all the individuals and organisations which have made this research possible. I would like to thank Professor Manuel A. Patarroyo for his unwavering support throughout and patience in helping me achieve this research's objective. Professor Patrick Van Dijck welcomed me in the MCB laboratory, supported me and instructed me in the world of mycological research, thereby successfully leading to the development of the work and always believed in the project. Professor Johan Thevelein also received me in the MCB lab and enabled this valuable experience. Professor Arley Gomez supported me during the first phase of the project thereby ensuring that it would start. I would also like to thank my supervisor in Leuven, Dr. Alessandro Fiori; his comments and questions always represented a source of instruction and support in developing the work. I would also like to thank Dr. Soňa Kucharíková for her advice and practical orientation regarding laboratory work and her example of discipline and enthusiasm regarding daily laboratory work and Dr. Hélène Tournu for valuable discussion and practical lessons when developing the research as well as her generous attitude to life.

I would especially like to thank Dr Beatriz Gomez and Dr Claudia Parra in Colombia for their dedicated support and generosity in providing continuity to that which began in Leuven

whose knowledge allowed me to include valuable elements in the development of the work. I would also like to acknowledge the valuable opportunity I had to meet Dr. Angela Restrepo and present the work to her and also receive valuable and appreciated feedback.

I would like to thank Deborah Syes, Ilse Palmans and Tom Adriani from the MCB laboratory for their support in the laboratory, my companions on the PhD programme Yuke and Fréderique for sharing agreeable moments during the work, as well as acknowledging and thanking Dr. Francoise Dumortier, Dr María Foulquie and Dr Marta Rubio at MCB and Evy, Catherine, Renata, Nico, Jan, Hilde and Leni for the always warm and friendly welcome in the MCB laboratory. I would like to thank FIDIC and recognise its valuable contribution towards finishing this work, as well as thanking Marina Muñoz and Diego Garzón for their technical support and Jason Garry for translating and revising the manuscripts.

I would like to thank the members of the PhD Candidacy Jury, Prof. Dr. Patrick Van Dijck, Prof. Katrien Lagrou, Dr. Alessandro Fiori and Dr.Angela Pinzón for their valuable support and comments thereby contributing towards orientating the work.

It is equally important to thank the Universidad del Rosario; I would like to acknowledge members of the grant committee, Dr. Leonardo Palacios, the dean of the School of Medicine and Health Sciences, Dr. Luisa Mateus from Sciences Faculty and Dr. Catalina Latorre in the Public Health Department for their form support, as well as ERACOL for the valuable opportunity provided me through its Erasmus Mundus programme regarding my professional development, just as that represented by KU Leuven, VIB in Flanders and FIDIC in Colombia.

I would like to thank my wife María Fernanda, children Samuel and María Camila, my parents Antonio and María Sofía, my adopted parents in Flanders Erik and Odette, my family and those in my adopted family in Flanders, as well as the community of San Miguel in Leuven for accompanying me, supporting me and making this experience the real treasure which it has been!

“**Man meets God behind every door science can open**”

Albert Einstein (1879-1955)

Contents

Summary

Candida albicans is the pathogenic fungus most frequently compromising patients in a hospital setting; its versatility in adapting to a host has enabled it to colonise their digestive tracts, genitourinary tracts and skin. Infection caused by this fungus represents a diagnostic challenge for doctors regarding their patients and represents high costs for health systems.

Healthcare technology has made advances aimed at improving the lifespan of critically ill patients; however, such patients become potential hosts for *C. albicans* as a successful commensal and pathogen of humans. Moreover, the speed at which new antifungal drugs are developed does not match up with the speed at which new reduced-susceptibility patterns to existing drugs are identified**.** The antifungal armamentarium has thus become reduced regarding this dimorphic yeast's adaptability as it has become resistant and increased the degree of difficulty concerning a critically ill patient's successful treatment.

C. albicans represents a diagnostic challenge in clinical practice since it is a commensal microorganism whose transformation into a pathogen remains partly unknown; moreover, its adaptation to a hospital environment poses an additional problem. However, advances in mycological techniques, proteomics for diagnosis and molecular biology can provide

diagnostic criteria for a better understanding of this dangerous pathogen threatening the critically ill patients' lives in hospitals worldwide.

Echinocandins are the latest antifungal molecules used in the systemic treatment of candidiasis. The medical and scientific community has recently decided to adjust echinocandin clinical breakpoint values for defining *C. albicans* pathogenic isolates' susceptibility or resistance values. It has been seen that the echinocandin resistance value in the standard Clinical and Laboratory Standards Institute (CLSI) minimum inhibitory concentration (MIC) test has been lowered in just seven years from ≥2μg / mL to ≥0.5 μg / mL.

Echinocandins represent an effective therapeutic option regarding *C. albicans* as such fungicides have a low toxicity profile. Their therapeutic target is the Fks1 protein and work by Angiolella *et al*., (2008) on laboratory strains showed that mutations in the *FKS1* gene encoding this protein have been associated with phenotypical changes determining increased virulence arising from such antifungals' resistance.

This PhD thesis was thus aimed at describing the pattern of a sample of *C. albicans* nosocomial isolates collected from third-level hospitals in Bogota, Colombia. It involved assessing echinocandin susceptibility patterns through standardised CLSI susceptibility tests using the latest two versions (M27-A3 and M27-S4), reduced susceptibility patterns being found in this sample. It also involved using two versions of CLSI for checking the resistance to micafungin of a nosocomial isolate which not been exposed to this antifungal.

Phenotypical changes explaining these *C. albicans* clinical isolates adaptation to cell wall stress caused by caspofungin were evaluated based on findings and information from the relevant literature. MALDI TOF-MS was used for discriminating and grouping *C. albicans* clinical isolates whose morphological and physiological patterns and the expression of genes involved in cell wall stability, outlining *C. albicans* species' atypical variability in this set of nosocomial microorganisms.

A correlation between nosocomial *C. albicans*-infection and the clinical outcome was observed, showing that patients having significant comorbidity needed careful nutritional assessment, as malnutrition was one of the factors significantly associated with mortality regarding this infection. This was identified as clinical diagnosis during hospital stay, in addition to finding that most atypical isolates came from critical patients whose management should be done in an intensive care unit (ICU).

This thesis thus describes the atypical pattern of a group of *C. albicans* nosocomial isolates leading to an association between phenotypical traits concerned with echinocandin tolerance and changes in these microorganisms' morphology and physiology regarding selective pressure factors in an antifungal-mediated hospital setting. Evaluation by molecular biology, conventional mycology and proteomics' tools should contribute towards constructing more accurate local epidemiology for decision-making regarding the management and control of hospital infection by this fungus.

Samenvatting

Candida albicans is de pathogene schimmel die het vaakst patiënten in een ziekenhuisomgeving treft. Deze schimmel heeft zich tijdens zijn evolutie vlot aangepast aan zijn gastheer waardoor hij in staat is om diens spijsverteringsstelsel, urogenitaal stelsel en huid te koloniseren. Infecties veroorzaakt door deze schimmel is een diagnostische uitdaging voor artsen en vormen een zware kost voor de gezondheidszorg.

De vooruitgang van de geneeskunde zorgt ervoor dat de toestand van ernstig zieke patiënten stabiel kan gehouden worden, maar dit maakt van deze patiënten ideale gastheren voor *C. albicans*. Door het feit dat schimmels ook eukaryoten zijn is er ook maar een beperkt arsenaal aan antischimmelproducten. Bovendien treedt er steeds meer en meer resistentie op tegen de huidige antischimmelproducten zodat het moeilijker wordt om *C. albicans* infecties te bestrijden.

De eigenschappen die de gist toelaten zich aan te passen aan een ziekenhuisomgeving blijven onbekend. Dit is grotendeels te wijten aan problemen in de diagnose, aangezien de stap van commensaal tot pathogeen nog gedefinieerd moet worden. Vooruitgang in mycologische technieken, proteomica voor diagnose en moleculaire biologie kunnen diagnostische criteria aanleveren voor een beter begrip van deze gevaarlijke ziekteverwekker die het leven van ernstig zieke patiënten in ziekenhuizen wereldwijd bedreigt.

De medische en wetenschappelijke gemeenschap heeft recent besloten om de klinische breekpuntwaarden voor één van de nieuwe antischimmelproducten, de echinocandines, aan te passen om de gevoeligheid of resistentiewaarden van *C. albicans* isolaten te definiëren. Echinocandines zijn de laatste antifungale moleculen die gebruikt worden in de systemische behandeling van candidiasis. Door de nieuwe regelgeving en bepaald via de standaard "Clinical and Laboratory Standards Institute (CLSI)" procedure, spreken we van resistentie tegen echinocandines wanneer de minimum inhiberende concentratie (MIC) ≥0.5 μg / mL is en niet meer ≥2μg / mL zoals voorheen.

Echinocandines vertegenwoordigen een effectieve therapeutische optie wat betreft *C. albicans* aangezien deze schimmeldodende moleculen een laag toxiciteitsprofiel hebben. Hun therapeutisch doel is het Fks1 proteïne en in het werk van Angiolella *et al*., (2008) werd duidelijk aangetoond dat dat mutaties in het *FKS1*-gen geassocieerd worden met een verhoogde resistentie tegen echinocandines. Dit proefschrift was dus gericht op het beschrijven van het gedrag van een steekproef van *C. albicans* nosocomiale isolaten verzameld uit tertiaire ziekenhuizen in Bogota, Colombia. We hebben de echinocandine gevoeligheidspatronen bepaald door middel van gestandaardiseerde CLSI gevoeligheidstesten, door gebruik te maken van de laatste twee versies (M27-A3 en M27 - S4), aangezien verminderde gevoeligheidspatronen gevonden werden in deze steekproef. We hebben beide CLSI methoden ook uitgevoerd om de resistentie van een nosocomiale isolaat die niet blootgesteld werd aan deze antifungaal tegen micafungine, één van de echinocandines, na te gaan.

Fenotypische veranderingen als gevolg van de celwand stress veroorzaakt door caspofungine, werden geëvalueerd op basis van de bevindingen en informatie van de relevante literatuur. MALDI TOF-MS werd gebruikt voor het onderscheiden en groeperen van klinische isolaten wiens morfologische en fysiologische patronen en expressie van genen betrokken in celwand stabiliteit, aanwijzingen geven voor de aanwezigheid van atypische variabiliteit van de *C. albicans* soort in deze set van nosocomiale micro-organismen.

De klinische correlatie uit deze bevindingen toonde aan dat patiënten met significante comorbiditeit zorgvuldige voedingsbeoordeling vereisen, aangezien ondervoeding één van de factoren was die significant geassocieerd wordt met mortaliteit wat betreft deze infectie. Dit werd geïdentificeerd als klinische diagnose tijdens ziekenhuisopnames, naast de bevinding dat de meest atypische isolaten afkomstig zijn van kritieke patiënten waarvan het beheer gedaan zou moeten worden in een intensieve zorg-afdeling (ICU).

Dit proefschrift beschrijft dus het atypische gedrag van een groep *C. albicans* nosocomiale isolaten die tot een associatie leiden tussen fenotypische echinocandine-tolerantiekenmerken en veranderingen in de morfologie en fysiologie van deze micro-organismen wat betreft selectieve drukfactoren in een antifungaal gemedieerde ziekenhuisomgeving. Evaluatie door moleculair biologische technieken, conventionele mycologie en proteomica-tools moeten bijdragen tot het bekomen van meer accurate lokale epidemiologie voor besluitvorming over het beheer en de controle van ziekenhuisinfecties door deze schimmel.

List of abbreviations

AB	Antibiotic.
ANOVA	Analysis of variance.
ATCC	American type culture collection.
BAL	Bronchoalveolar lavage.
BDAL	Bruker database library.
BMD	Broth micro-dilution test.
BSI	Blood stream infections.
cAMP	Cyclic adenosine monophosphate.
CBP	Clinical breakpoints.
CDC	Centers for disease control and prevention.
cDNA	Complementary DNA.
CFW	Calcofluor white.
CHS	Chitin synthase.
CI	Confidence interval.
CLSI	Clinical laboratory standards institute.
CSF	Caspofungin.
Ct	Cycle threshold.
CVC	Central venous catheter.
CWI	Cell wall integrity.
DMSO	Dimethyl sulfoxide.
DNA	Deoxyribonucleic acid.

EUCAST	European Committee for Antimicrobial Susceptibility Testing.
FBS	Fetal bovine serum.
FCS	Fetal calf serum.
FDA	Federal Drug Administration.
FLU	Fluconazole.
G	Geometric mean.
gDNA	Genomic DNA.
GPI	Glycosylphosphatidylinositol.
GTP	Guanosine triphosphate protein.
HAT	Histone acetyltransferase.
HDAC	Histone deacetylase.
HIV	Human Immunodeficiency Virus.
HOG	High-osmolarity glycerol.
HS	Hot spot.
I	Intermediate.
IC	Invasive candidiasis.
ICU	Intensive care unit.
ID	Identification.
MALDI	Matrix-assisted laser desorption/ionization.
MALDI TOF-MS	Matrix-assisted laser desorption/ionization Time of Flight Mass Spectrometry.
MAPK	Mitogen-activated protein kinase pathway.
MCF	Micafungin.

MDS	Metric multi-dimensional scaling.
MIC	Minimum inhibitory concentration.
MSP	Main spectrum.
MOPS	Morpholinepropanesulfonic acid buffer.
MTL	Mating type-like.
NCCLS	National Committee for Clinical Laboratory Standards.
OD	Optical density.
OPC	Oropharyngeal candidiasis.
ORF	Open reading frame.
PCR	Polymerase chain reaction.
PF	Peritoneal fluid.
Pir	Proteins having internal repeats.
PKA	Protein kinase A.
PKC	Protein kinase C.
qPCR	Quantitative PCR.
R	Resistant.
RNA	Ribonucleic acid.
ROC	Curve receiver operating characteristic curve.
rRNA	Ribosomal RNA.
RT	Real time.
Saps	Cell surface-associated proteases.
SC	Superficial candidiasis.
S	Susceptible.

TPN	Total parenteral nutrition.
UPGMA	Unweighted pair group method with arithmetic mean.
UTI	Urinary tract infection.
VVC	Vulvovaginal candidiasis.
YNB	Yeast nitrogen base.
YPD	Yeast peptone dextrose, a nutrient – rich medium.

List of genes

ADA2	Transcriptional ADAptor
ALS3	Agglutinin-Like Sequence
CAS5	CAspofungin Sensitivity
CDR1	Candida Drug Resistance; Multidrug transporter of ABC superfamily
CDR2	Candida Drug Resistance; Multidrug transporter, ATP-binding cassette (ABC) superfamily
CNB1	Regulatory subunit of calcineurin B (Ca[2+]-calmodulin-regulated S/T protein phosphatase)
CRH11	Congo Red Hypersensitive; GPI-anchored cell wall transglycosylase
CRZ1	Calcineurin-Responsive Zinc finger; Calcineurin-regulated C2H2 transcription factor
DFG5	Defective for Filamentous Growth; N-linked mannoprotein of cell wall and membrane
ECE1	Extent of Cell Elongation; Hypha-specific protein
EFG1	Enhanced Filamentous Growth; bHLH transcription factor
ERG5	ERGosterol biosynthesis; Putative C-22 sterol desaturase
FKS1	beta-1,3-Glucan Synthase Catalytic subunit; Essential beta-1,3-glucan synthase subunit
GAT2	Biofilm ReGulator (BRG1); Transcription factor
GPR1	G-Protein coupled Receptor; Plasma membrane G-protein-coupled receptor of the cAMP-PKA pathway
GSL2	Glucan Synthase-Like; Protein similar to beta-1,3-glucan synthase
HST3	Histone H3K56 deacetylase; reduced copy number increases opaque cell formation
HWP1	Hyphal cell wall protein
MID1	Mating pheromone-Induced Death
MKC1	MAP Kinase from *C. albicans*; MAP kinase
NRG1	Negative Regulator of Glucose-repressed genes

PGA13	Predicted Gpi-Anchored; GPI-anchored cell wall protein involved in cell wall synthesis
PGA23	Predicted Gpi-Anchored; Putative GPI-anchored protein of unknown function
PGA31	Predicted Gpi-Anchored; Cell wall protein
PGA4	Predicted Gpi-Anchored; GPI-anchored cell surface protein
PGA62	Predicted Gpi-Anchored; Adhesin-like cell wall protein
PHR1	PH Responsive; Cell surface glycosidase
PNG2	Peptide N-Glycanase; Putative peptide:N-glycanase; gene has variable numbers of 12-bp repeats
RLM1	Resistance to Lethality of MKK1P386 overexpression
RTT109	Regulator of Ty1 transposition; Histone acetyltransferase
SKO1	Suppressor of Kinase Overexpression; bZIP transcription factor
SOD5	Cu-containing superoxide dismutase
SSN6	Cytochrome C; Functional homolog of *S. cerevisiae* Cyc8/Ssn6
SSR1	Beta-glucan associated ser/thr rich cell-wall protein with a role in cell wall structure
TEC1	Transposon Enhancement Control
TPS2	Trehalose-6-phosphate (Tre6P) phosphatase
TUP1	dTMP-Uptake; Transcriptional corepressor

List of Figures

List of Tables

Chapter 1 - Introduction

Candida albicans is the major human fungal pathogen. It is a ubiquitous organism in nature and is a normal commensal in the human digestive tract, the female genital tract and the skin; however, Candida can cause oral and vaginal thrush, mucosal and systemic infections (invasive candidiasis, blood stream infections) in immunocompromised patients or those at risk [1]. Fungal infection in the nosocomial environment is mainly caused by *Candida* spp., *Candida albicans* being the species most frequently involved in this group of infections [2].

Several studies have shown that nosocomial candidaemia frequency increased by 500% from 1980 to 1989 in large teaching hospitals and by 219% and 370% in small teaching and large nonteaching hospitals, respectively [3]. This trend continued throughout the 1990s when Candida species were reported to be the sixth most common nosocomial pathogen and the fourth most common bloodstream pathogen. This trend was confirmed in 2004 in one of the largest multicentre studies performed to date [4].

According to previous studies, the mortality attributed to nosocomial candidaemia was 49% with a 95% CI of 38-60% in 2003 and the infection was associated with a much higher crude attributable mortality than expected from the underlying disease alone [5]. Candidaemia is a major problem in adult and neonatal patients' intensive care units (ICU), where an estimated 33-55% of all episodes occur [6]. Regarding Colombia, 33% mortality rates have been reported in epidemiological outbreaks of *Candida* in third-level institutions [7]. A study

carried out by our group over a six-month period evaluated *Candida* spp. strains isolated from ten hospitals in Bogotá, Colombia (2006), giving 40.22% associated mortality rate in ICUs and 28.95% in hospital wards. This study provided fundamental information about the status of nosocomial infection by this fungus in a representative sample of Colombian hospitals; however, the resistance mechanism and its relationship with virulence have not been studied to date. This project has thus been aimed at understanding the main factors associated with resistance and virulence when using new antifungal options such as the echinocandins.

Systemic antifungal agents are indicated for the treatment of invasive candidiasis and candidaemia; however, azole (fluconazole) use as prophylaxis in ICUs is inducing resistance [8]. The ARTEMIS DISK Global Antifungal Surveillance Study, lasting from June 1997 to December 2003, was a 6.5-year analysis of fluconazole and voriconazole susceptibility by Candida and other yeast species. This study revealed 1.3% resistance to fluconazole and 1.0% to voriconazole, these antifungals' resistance in Colombia being 6.1% and 4.0%, respectively [9]. Despite the low incidence of *in vitro* echinocandin resistance rates observed in the epidemiological surveillance programme, some cases related to treatment failure in patients receiving these drugs have been reported since 2005, displaying cross-resistance to all drugs belonging to this group; another study carried out in Paris (France) showed a 0.4% echinocandin resistance incidence for *Candida* spp. [10-12]. Regarding antifungal agents belonging to the polyenes group (such as amphotericin B), the main problem concerning their use has been related to nephrotoxicity [13]. The echinocandins are thus the only new class of antifungal drugs of choice in clinical practice against candidaemia and invasive candidiasis.

Whilst azole resistance has been widely studied, the underlying resistance mechanisms for echinocandins are still being investigated since their use in medical environments is relatively recent [14]. The main mechanism involved in echinocandin resistance described to date has been the presence of point mutations in the *FKS1* gene, but its relationship with virulence, tolerance and reduced susceptibility still remains unclear [15].

Sorgo *et al*., 2011 showed in an interestingly behaviour related with exposition to fluconazole that increases the regulatory activity in order to keep strength factor in *Candida albicans'* cell wall [16]. This behaviour could represent an impact in reduced susceptibility to antifungal agents such as echinocandins regarding its action mechanism.

The present study has thus been aimed at providing new information from a valuable clinical sample about the role of Fks1 and its relationship to tolerance, reduced susceptibility and virulence in nosocomial infection caused by *Candida albicans.*

1.1. *Candida albicans* biology

The Candida genus consists of around 200 species; 30 of them have been identified as being pathogenic for humans. *Candida albicans* has been the main pathogen described for this group [17]. It is a hemiascomycete which diverged from *Saccharomyces cerevisiae* 840 million years ago [18]; it is a commensal, yeast, pleomorphic fungus which is present in the general population, preferably on the skin, in the oral mucosa, gastrointestinal tract and the genitourinary tract [1].

1.1.1. *C. albicans* taxonomy

C. albicans taxonomy has become more complex recently due to the appearance of closely-related species, in turn, leading to greater difficulty for clinical laboratories in providing precise identification [19]. Identification criteria have recently included molecular biology and proteomics techniques, such as genetic markers or spectrometry patterns like those obtained by MALDI-TOF MS [17]. The importance of identifying *Candida* species and/or *C. albicans*-related species lies in their pathological implications and epidemiological representation [20].

C. albicans belongs to Superkingdom: Eukaryota; Kingdom: Fungi; Phylum: Ascomycota; Class: Saccharomycetes; Order: Saccharomycetales; Genus: *Candida*; Species: *Candida albicans*. *Candida albicans* has four cell morphologies known as blastospores (also known as yeast), pseudohypha, hypha and chlamydospores; recognising these cell forms is important as taxonomic classification criteria (**Figure 1**), chlamydospore formation, carbohydrate assimilation, being most relevant for distinguishing between related species [21-23] . Efg1 is

required for hypha formation in presence of serum, neutral pH, 5% CO2 (the partial pressure of CO2 in bloodstream) and N-acetyl D- glucosamine, additional hypha inducing signals and the transcriptions factors are summarized in (**Figure 2**). Hypha formation is considered one of the main factors related with the virulence trait in *Candida albicans* and *EFG1*, activating the cyclic AMP pathway, is considered one of the main hypha specific genes involved in this phenotypic trait. Another hypha specific gene is *CPH1;* its pathway is based on MAPK signalling pathway. *RAS1* stimulates both cAMP and MAPK pathways (**Figure 2**) [23].

The minor species described to date for *C. albicans* have been, in order, *C. dubliniensis* described by Sullivan in 1995 in patients having a background of HIV and oropharyngeal candidiasis (OPC) and its presence is more frequent in cystic fibrosis patients [24] and the related species, *C. africana*, described by Tietz in 2001 in patients having a background of vulvovaginal candidiasis (VVC) [21, 25].

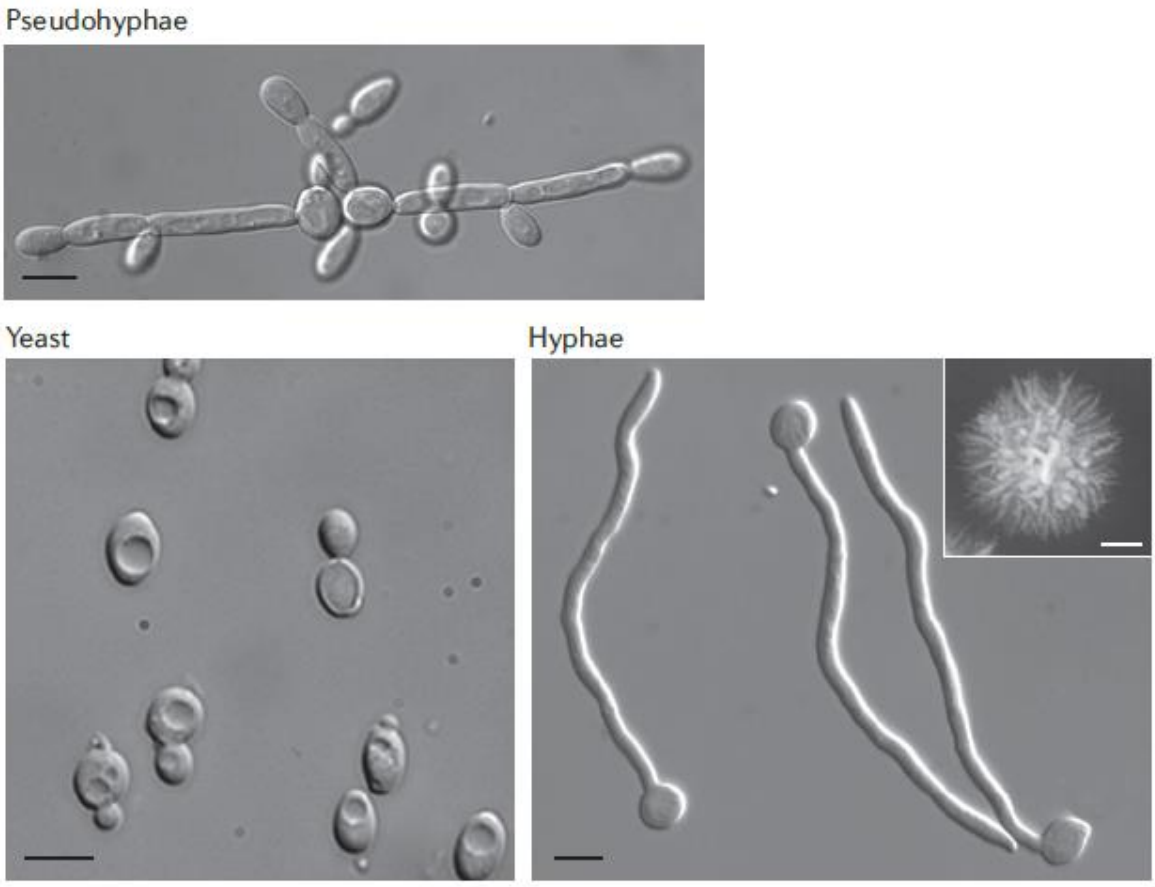


Figure 1. Morphology of yeast, hyphae and pseudohyphae from *C. albicans* (Sudbery, 2011).

Inset of the hyphae panel shows the hyphal colony after 5 days growing on Spider media. Scale bars in the main panels represents 5µm and in the inset panel 1mm.

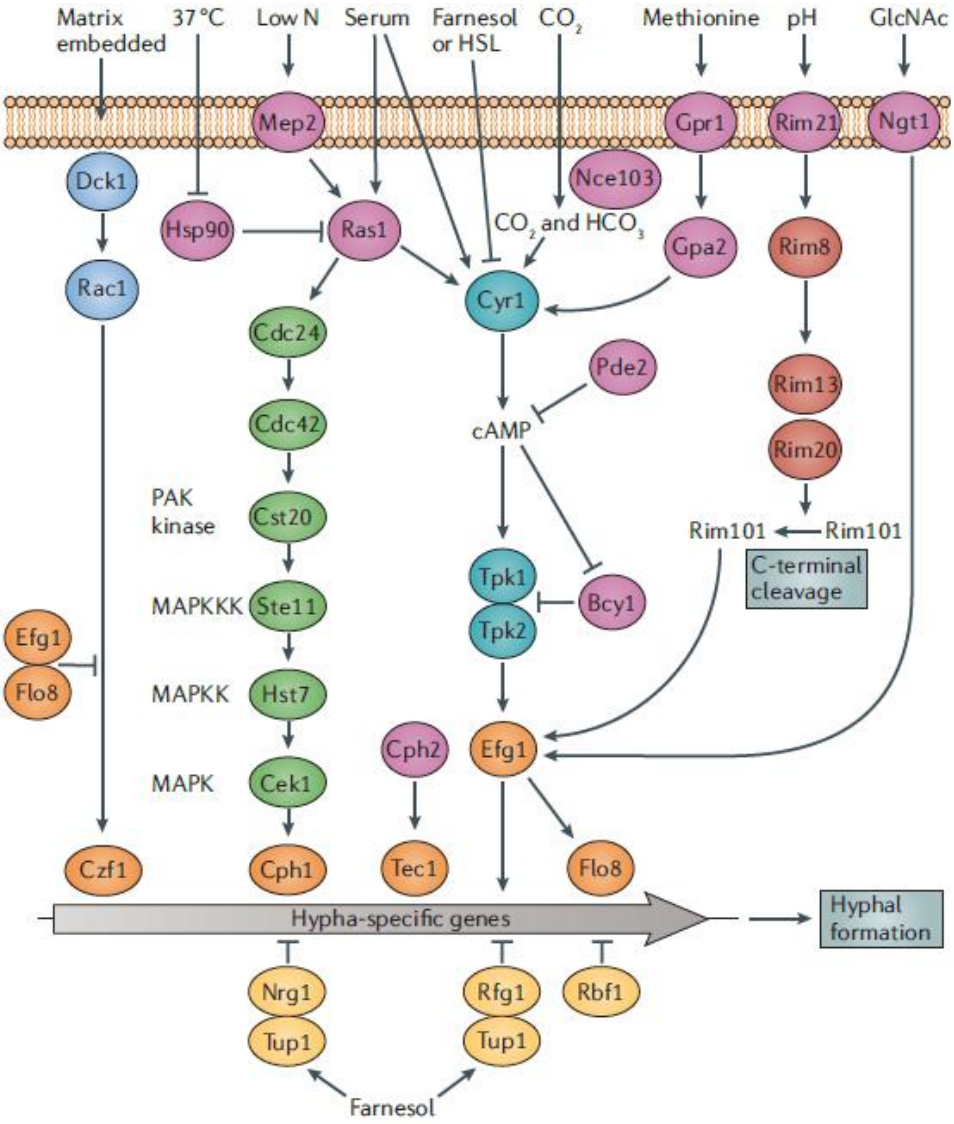


Figure 2. Signal transduction pathways leading to expression of hypha-specific genes (Sudbery, 2011).

Several environmental stimuli turn on downstream pathways to activate a panel of transcriptions factors. The cyclic AMP dependent pathway plays a major role, targeting the transcription factor Efg1. Adenylyl cyclase integrates multiple signals in both Ras-dependent and Ras-independent ways. Tup1 gives the negative regulation by targeting the promoters of hypha specific genes through DNA binding proteins such as Nrg1 and Rfg1.

1.1.2. The *Candida albicans* genome

Considering *C. albicans*' medical importance, it was one of the first eukaryotic pathogens to be selected for whole genome sequencing **(**SC5314 reference strain**)**; its size was estimated as being 14.3 Mb, having eight chromosomes ranging in size from 1.03 Mb to ~ 4.3Mb (numbered 1 to 7: chromosome number 1 being the largest and 7 the smallest). Its R chromosome has ribosomal DNA, its size varying from 2.6 Mb to 3.43 Mb [26-28]. This fungus' genetic grouping is determined by the CTG codon which translates serine instead of leucine, thereby placing this specie in a specific clade (the CTG clade), together with other diploid fungi [29]. According to the Candida Genome Database (www.candidagenome.org) (last accessed on May 19th 2015), the functions of 24.83% of the 6,218 annotated ORFs had been verified, 2.44% were doubtful and 72.72% had not been characterised [30].

Regarding the exchange of genetic material in *C. albicans* and its reproduction, this microorganism was initially considered asexual; however, the finding of a mating type-like (MTL) locus has led to a parasexual cycle supported by a robust system being described, in which mating between diploid MTL a and α strains leads to the production of tetraploid a/α strains. Such mechanism is regulated by a phenotypic change in homozygote MTL strains (a/a and α/ α), which can reversibly change into two inherited states known as white cells and opaque cells. The opaque cells are the only form that allows pairing/mating. *C. albicans* tetraploid strains return to a diploid state by losing chromosomes; it is considered that this alternate mechanism described in *C. albicans* represents an evolutionary mechanism regarding adaptation to warm-blooded hosts [31].

1.1.3. The *Candida albicans* cell wall

The *C. albicans* cell wall provides the fungus with structure and interaction with its environment, as well as constituting a permeable barrier guaranteeing protection and form for the cell. This organ also has to do with the fungus adapting to different forms of life, in this case, the transition from yeast to hypha which is key for virulence [32].

Regarding cell wall components, 80%-90% are polysaccharides (β-glucans, chitin and mannoproteins), 5%-20% proteins (cell wall proteins) and 1%-7% lipids [33, 34] (**Figure 3**).

Polysaccharides in the *Candida albicans* cell wall

- Glucans: β 1-3 glucan is the molecule having the greatest presence in the wall, consisting of D-glucose molecules assembled linearly by β 1-3 glycosidic bonds distributed linearly across the cell wall. Together with chitin, it is responsible for cell wall rigidity. β 1-6 glucan is a very branched polymer fulfilling an essential function in the cell wall by being an interconnector between β 1-3 glucan and glycosylphosphatidylinositol (GPI)-anchored proteins;
- Chitin: is one of the components frequently found in the cell wall of fungi but to a lesser extent than glucans. It is a linear polymer formed by N-acetyl-D-glucosamine molecules bound by β 1-4 bonds, thereby providing the fungus' cell wall with rigidity; and
- Mannan: this polymer is located on the external part of the cell, also known as the phosphopeptidomannan complex consisting of mannose homopolymers having 3%

to 5% proteins and 1-2% phosphate. This polymer provides characteristic hydrophobicity for the cell surface [32].

Candida albicans cell wall proteins

- Proteins having internal repeats (Pir): proteins bind to β 1-3 glucan by an alkali bond without binding to β 1-6 glucan; and
- Glycosylphosphatidylinositol (GPI)-anchored proteins: 90% of these proteins are bound to β 1-3 glucan via β 1-6 glucan; the rest bind to chitin via β 1-3 glucan. GPI proteins might be implicated in cell wall biosynthesis and also in cell wall remodelling. These proteins might determine hydrophobicity or antigenicity as well as being considered as having to do with to this fungus' adherence and virulence properties [35]. Previous studies in the *S. cerevisae* yeast model have estimated that there are more than 115 GPI proteins and more than 65% of these proteins have an unknown function. Regarding *C. albicans*, several studies have been related to a GPI functional role for this group of proteins, describing an interesting function related to cell wall stability. A systematic and functional study has described some mutant strains related to increased or decreased susceptibility against caspofungin or having defective growth condition under cell stress. Some genes involved in action related to cell wall maintenance would be *SOD5*, *PGA62*, *PGA31*, *PGA13* and *PGA23* [36-38].

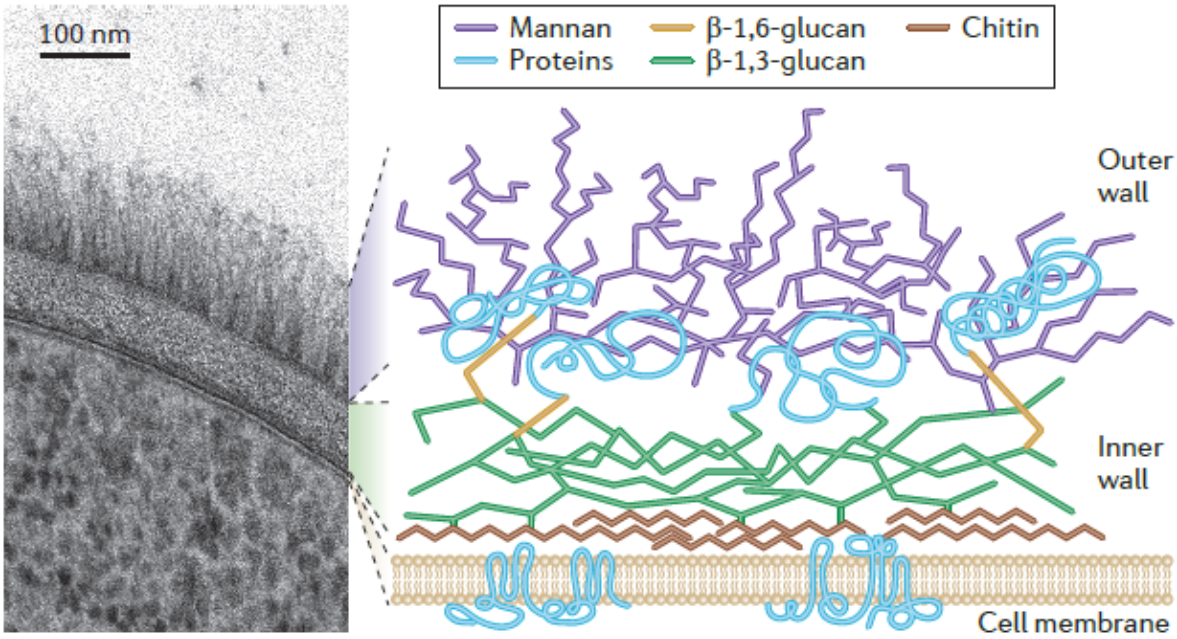


Figure 3. *Candida albicans* cell wall structure of the (Gow *et al*., 2012).

Transmission electron micrograph showing the *C. albicans* two-layered cell wall, the internal layer provides cell shape and strength through chitin polysaccharides with β-1,3-glucan. The outer layer consists of mannose polymers that are covalently associated with proteins to form glycoproteins. The glycosylphosphatidylinositol proteins are located in the outer wall and linked to the skeleton by flexible structures of β-1,6-glucan.

1.2. The epidemiology of *Candida*-associated infection

Candida spp. have been the fourth most common cause of bloodstream infections in the USA for more than a decade; the mortality rate associated with candidaemia ranges from 40% to 49% [5, 6]. Candidaemia incidence, according to population-based studies, is around 1.9 to 11 cases per 100,000 inhabitants in Europe, 6.0 to 24 cases per 100,000 inhabitants in the USA and 2.8 per 100,000 inhabitants in Canada [6]. The ARTEMIS DISK Global Antifungal Surveillance Study (from 1997 to 2003), which described the distribution of Candida species collected from invasive candidiasis (IC) in 127 medical centres from 39 countries, revealed that *C. albicans* was the most common species causing IC (62% distribution regarding the

total sample) [9]. Regarding infection pattern in tertiary referral centres, a retrospective study has shown an overall *Candida spp*. incidence rate of 1.5 / 1,000 hospital admissions, where *C. albicans* represented the most commonly isolated species (48%). Risk factors associated with mortality were: older age, hospitalisation in an ICU or in a medical ward rather than a surgical ward, infection by *C. albicans* rather than other species, the presence of septic shock, pneumonia or acute renal failure, the presence of a solid organ tumour or a chronic pulmonary disease [39].

It has been estimated that 2.5% to 10% of all patients admitted to US hospitals will develop a nosocomial infection. Blood stream infections (BSI) represent 10% of all nosocomial infections, 8% of BSI being caused by *Candida* species; this represents 7,000 to 28,000 nosocomial candidaemia cases annually. If the crude mortality rate is around 40%, then 2,800 to 11,200 deaths each year may be associated with nosocomial candidaemia just in the USA [6]. Even though *C. albicans* leads to infections identified in hospitalised patients, evidence has been presented regarding the emergence of non-albicans species [40] as being pathogens of medical interest. Concerning north-America and Europe, *C. glabrata* is the second species in frequency, whilst *C. parapsilosis* in Latin-America and *C. tropicalis* in Asia must be highlighted [40-42]. The emergence of non-albicans species has also been associated with the widespread use of antifungal agents such as azoles and echinocandins [40].

The economic impact of systemic fungal infections on the US public health system was $2.6 billion in 1998, the average attributable cost per patient being $31,200 [43]. According to a 2005 Belgian health system report, blood stream infections led to an additional €13,582 cost

per patient [44] and cost per infection in US$ was 32,810 according to cost impact studies, when prolonged length of hospital stay was considered [45].

1.3. Clinical presentation

IC involves clinical spectra such as candidaemia, disseminated candidiasis with deep organ involvement, endocarditis and meningitis. The clinical manifestations are nonspecific but the most common symptom is fever having acute or insidious onset (even present during antibiotic therapy) which can be continuous or intermittent [46]. Different causes of infection prevail; deeply invasive candidiasis may be due to haematogenous seeding. Deep oesophageal infection may result from penetration by organisms from surface oesophageal erosions. Joint or deep wound infection can originate from the contiguous spread of organisms from the skin and kidney infection from the catheter-initiated spread of organisms through the urinary tract. Infection of intra-abdominal organs and the peritoneum can be caused by perforation of the gastrointestinal tract and gallbladder infection from retrograde migration of organisms from the gastrointestinal tract into the biliary drainage system [47, 48].

Mucosal candidiasis of the oral, gastrointestinal and vaginal cavities represents the most common form of superficial candidiasis (SC). The clinical manifestations are related to anatomical location; for example, patients suffering from oral candidiasis complain of burning pain and odynophagia whilst those suffering from chronic atrophic stomatitis or denture stomatitis refer to having burning local pain. The symptoms include dysphagia, odynophagia and retrosternal chest pain in cases of oesophageal candidiasis whilst

vulvovaginal candidiasis involves symptoms such as vulvar pruritus, vaginal discharge, vaginal soreness, irritation, vulvar burning, dyspareunia and/or external dysuria [46].

IC involves risk factors such as parenteral nutrition, major abdominal surgery, diabetes, chronic renal insufficiency, the use of broad-spectrum antibiotics and a central venous catheter [49].

1.4. Nosocomial infection

Nosocomial infection is a localised or systemic condition which can be defined as an adverse reaction to the presence of an infectious agent or its toxins which was not present or incubating at the time of admission to hospital. For most bacterial nosocomial infections this means that the infection usually becomes evident 48 hours or more after admission. However, because the incubation period varies with the type of pathogen and, to some extent, with a particular patient's underlying condition, each infection must be assessed individually for evidence linking it to hospitalisation. Nosocomial infection diagnosis is based on some other principles such as clinical findings combined with laboratory test evidence and/or direct observation by a doctor or surgeon [50, 51].

A description of nosocomial infection caused by *C. albicans* refers to patients' critical conditions related to the motive for their hospitalisation, *C. albicans* having been identified as the specie having the greatest participation in IC and candidaemia. *Candida* spp. occupy third place regarding frequency of identification in blood stream infection, following *Staphylococcus aureus* and *Staphylococcus epidermidis* and tying with *Enterococcus* spp.;

however, it must be born in mind that disseminated candidiasis is found in 30% to 50% of the clinical autopsies for patients dying from sepsis whose haemocultures were reported as being negative. Candida infections are considered epidemiologically significant due to the more than 15-fold increase reported between 1970 and 1990, along with the pertinent costs involved [52, 53].

1.5 Antifungal treatment

Antifungal drugs have been developed since the 1950s; however, the armamentarium for treating *Candida*-related invasive infections may now be insufficient because of the increasing amount of patients having risk conditions, as well as resistance-associated therapeutic failure rates. Additional factors such as market availability, cost and toxicity reduce management options [54, 55]

1.5.1. Azoles

The agents approved for use regarding IC include the triazoles formed by fluconazole, itraconazole, posaconazole and voriconazole. Isavuconazole is a new molecule currently in global phase III clinical trials for IC. Triazoles block the synthesis of ergosterol (**Figure 4**), the main sterol in fungal membranes, by targeting lanosterol–14α-demethylase, also known as Erg11p or CYP51p. The effect related to this blockade consists of ergosterol depletion and changes in membrane permeability, changes in membrane bound proteins involved in cell wall synthesis, toxic sterol synthesis, resulting from Erg3p activity and 14α-methylated sterol accumulation. Evidence has also been presented that they act on cell wall structure. Some

authors have demonstrated the presence of compensatory responses similar to cell wall disrupting agents [16, 55].

1.5.2. Polyenes

Amphotericin-B was developed in the 1960s; presentations are currently available in liposomal and lipid complex to reduce collateral effects regarding renal function. The mechanism of action comes from a strong junction with ergosterol and causes pore formation having a fungicidal effect (**Figure 4**). A new discovery has been related to a mechanism of amphotericin cytocidal action, acting like a sponge and extracting ergosterol from phospholipid bilayers [55, 56].

1.5.3. Flucytosine

This is an antifungal agent acting on nucleic acids and protein synthesis. Flucytosine is a pyrimidine analogue which is converted to 5-fluorouracil which, once metabolised, becomes a substrate producing toxic nucleotides causing DNA disruption and protein synthesis (**Figure 4**). It is available for oral and intravenous administration; however, its availability is limited even though having been identified in 1957 and being used as an antifungal since 1968 [55, 57].

1.5.4. Echinocandins

Echinocandins are cyclic hexapeptides having N-linked acyl lipid side-chains. These are semisynthetic lipopeptides produced via the chemical modification of natural fungal products; for instance, caspofungin production from pneumocandin B_0 (*Glarea lozoyensis*),

micafungin (hexapeptide FR901370 from *Coleophoma empedra*) and anidulafungin (echinocandin B_0 from *Aspergillus nidulans*) [58]. This group of compounds is the newest class of antifungals to reach hospitals in decades and is active against clinically-relevant yeasts and moulds. Caspofungin, the first class member, received FDA approval in 2002, followed by micafungin in 2005 and anidulafungin in 2006. The echinocandins inhibit β–1,3-D-glucan synthase, an enzyme involved in fungal cell wall synthesis (**Figure 4**) [59]. Disrupting this polysaccharide results in the loss of cell wall integrity and severe wall stress for the fungi [60].

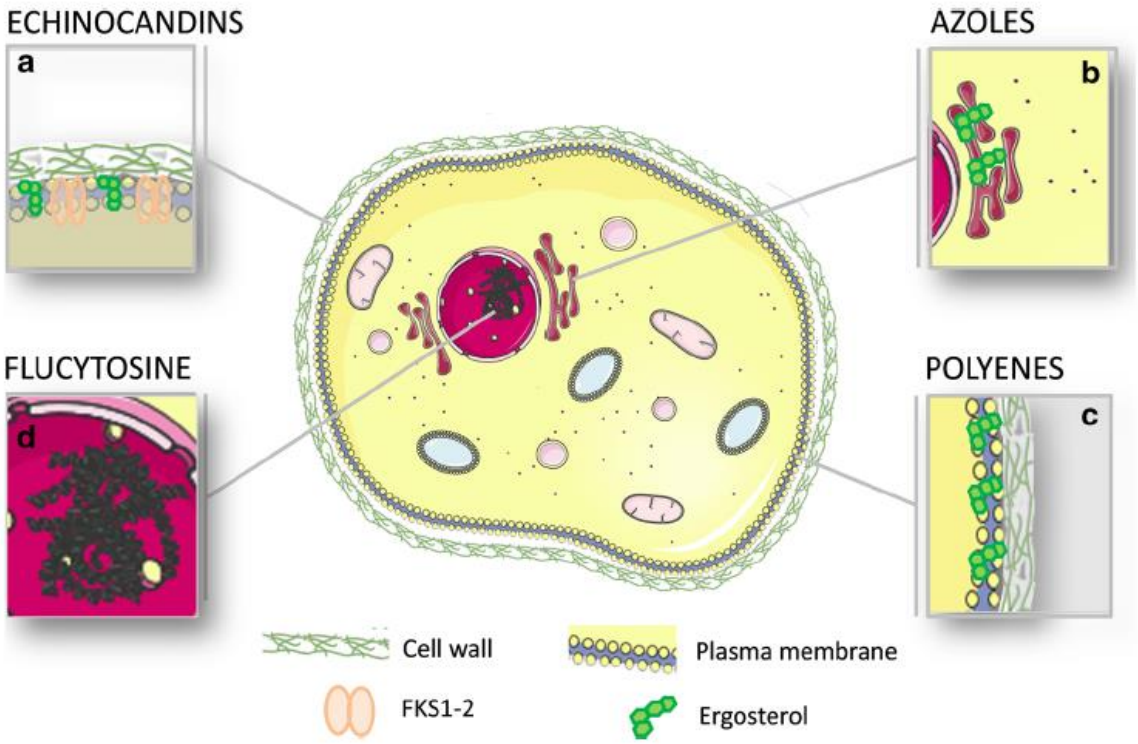


Figure 4. Targets and mechanisms of antifungal agents (Maubon *et al*., 2014).

a. Inhibition of Fks1p by Echinocandins, thus blocking β-1,3 glucan synthesis. b. Azoles blockage of 14α-demethylase results in an ergosterol depletion in the membrane and activation of Erg3p alternative pathway, leading to the synthesis of toxic sterol. c. Polyenes bind to the cell membrane ergosterol creating pores and aggregates, thus causing ion depletion. d. Flucytosine acts in the nucleus and inhibits DNA synthesis.

Members of the echinocandins group are concentration–dependent, non-competitive inhibitors of β–1,3-glucan synthase, an enzyme complex consisting of two subunits: Fks1 and Rho. Glucan synthase is involved in the synthesis of β–1,3-D-glucan, a polysaccharide consisting of 3 helically-entwined glucose polymers.

Despite substantial work aimed at elucidating the precise location where echinocandins bind to the glucan synthase enzyme complex, this question remains unsolved. Glucan is an essential fungal cell wall carbohydrate component, comprising 30%-60% of the fungal wall in Candida and Saccharomyces. Consequently, changes in cell wall characteristics can lead to osmotic instability and eventually to cell lysis. On the other hand, human cells do not contain β–1,3-D-glucan, so there is no toxicity for human cells [61].

According to the clinical guidelines for the management of candidiasis published by the European Society of Clinical Microbiology and Infectious Diseases in 2012, echinocandin prescription is recommended:

- As initial therapy for most adult non-neutropenic patients and neutropenic patients.
- As an empirical treatment for presumptive moderate to severe, or severe invasive candidiasis in non-neutropenic patients who have had recent azole exposure.
- As a first option in neutropenic patients at high risk of infection by *C. glabrata* or *C. krusei* prescribed with lipid formulation of amphotericin-B (LFAmB) and in neonatal candidiasis.

Echinocandins should be used with caution and are usually limited to situations where resistance or toxicity precludes the use of fluconazole or amphotericin-B deoxycholate (AmB-d) [62, 63].

Antifungal prophylaxis with fluconazole, posaconazole or caspofungin is recommended in patients suffering chemotherapy-induced neutropenia, whilst fluconazole, posaconazole or micafungin are recommended for stem cell transplant recipients having neutropenia or at risk of neutropenia [13, 63].

1.6. Drug resistance

Eukaryotic pathogens, such as fungi, represent a challenge since they are closer to humans than bacteria and viruses from the evolutionary point of view and there are thus less useful drug targets for selectively killing them. Fungi have emerged as a major cause of human disease, especially in immunocompromised individuals. Acquired fungal bloodstream infections have increased by 207%, being the major cause of death related to *Candida albicans* and *Aspergillus fumigatus* infection [64]. Indeed, there are limited therapeutic options and the echinocandins represent the only novel class of antifungals [14].

One of the main strategies for survival in hostile host environments is related to an ability to develop drug resistance which can be clinically defined by laboratory tests and molecular markers. From the clinical point of view, drug resistance is the evidence of treatment failure, despite appropriate drug therapy. Laboratory drug resistance is quantified by using a minimal inhibitory concentration (MIC) assay in which pathogen growth is measured with a series of

drug concentrations over a defined period of time according to standard protocols. From a molecular point of view, echinocandin resistance is defined as the presence of characteristic mutations in the glucan synthase 1 (*FKS1*) gene which has been firmly associated with resistance [59].

Resistance can be classified as primary resistance when the fungus is resistant prior to exposure to an antifungal agent, or secondary/acquired resistance when exposure to an antifungal agent induces drug resistance mechanisms [65].

1.6.1. Drug resistance mechanisms

Fungal responses against antifungal treatment are complex and their classification as susceptible or resistant is not easily achieved. Several molecular alterations enable fungi to survive exposure to antifungal drugs [64]; these can be divided into categories such as transport alterations, target alterations or amplifications, using compensatory pathways (cell stress response) and the presence of complex multicellular structures (biofilms) [66] (**Table 1** summarises such mechanisms):

1. **Transport alterations:** Experimental studies have shown that overexpression of CDR1 and CDR2 drug transporter genes in *C. albicans* clinical isolates reduced susceptibility to caspofungin and slightly increased susceptibility to micafungin in agar plate assays. Interesting studies have shown that the overexpression of the CDR2 gene results in resistance against caspofungin; such data has suggested that cross-resistance could be a consequence of multiple mechanisms acting at the same time [14, 67, 68];

2. **Target alterations:** These mechanisms have often been described regarding echinocandin resistance. Mutations in the enzyme, Fks1, targeted by the echinocandins are well characterised and result in resistance. Laboratory studies on both *C. albicans* and *S. cerevisae* have mapped two hot spot (HS) regions in the *FKS1* gene which are important for echinocandin resistance and correlate with higher MIC levels [15];
3. **Using compensatory pathways**: Addition of drugs results in the activation of stress response pathways. An important pathway that is activated is the calcineurin pathway, playing an important role since its inhibition in combination with cyclosporine has a synergistic effect with echinocandins. Hsp90 regulates calcineurin stability and its genetic compromise or pharmacological inhibition has a synergistic effect with echinocandins [14]. The G-protein, Rho1 and protein kinase C (PKC) play a specific role in the cellular stress response pathway, mediating resistance to echinocandins; the PKC cell wall integrity pathway has been implicated in upregulating chitin synthase (CHS) gene expression in response to β–1,3-D-glucan synthase inhibition by echinocandins [59]. Histone deacetylase (HDAC) Hst3 and histone acetyltranferase (HAT) Rtt109, have recently been shown to regulate acetylation in *C. albicans*. Deleting *RTT109* has conferred increased susceptibility to micafungin and caspofungin, as well as other genotoxic stresses, such as hydroxyurea and methyl methanesulphonate. Clinical *C. albicans* isolates are sensitive to nicotinamide, an inhibitor of the NAD^+-dependent HDAC complex, such as Hst3. Nicotinamide has been shown to reduce fungal kidney burden when used in mouse models with infection [14]; and
4. **The presence of complex multicellular structures (biofilms):** Several groups have shown that the Candida biofilm lifestyle has dramatically increased levels of resistance to the most commonly used antifungal agents. However, new antifungal agents, such as

the echinocandins and liposomal formulations of amphotericin-B, have shown increased activity against Candida biofilms [69]. It should be stressed that **CLSI** broth dilution techniques for antifungal susceptibility testing use planktonic populations and are unable to predict antifungal efficacy against Candida biofilms [70]. Several mechanisms may be responsible for Candida biofilms' intrinsic resistance, including the effects of the biofilm matrix on drug penetration, decreased growth rate and nutrient limitation, expression of resistance genes and the presence of "persister" cells. However, the current consensus is that biofilm resistance is a complex phenomenon which cannot be explained by one mechanism alone; instead, it is multifactorial and may involve different molecular resistance mechanisms [71].

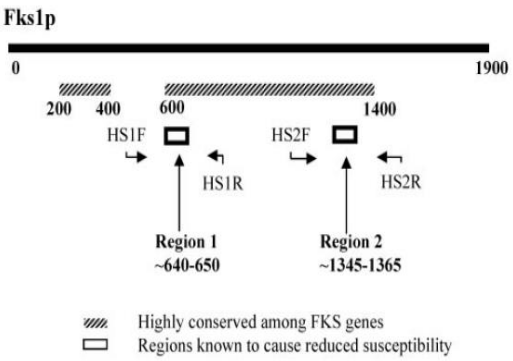
Table 1. Resistance mechanisms regarding *Candida albicans*

Drug resistance mechanism	**Type of mechanism for drug resistance**	**Observation**	**Reference**
Transport alterations	*CDR1*-*CDR2* overexpression (drug transporter)	Show echinocandin resistance	[59, 67]
Target alterations	Point *FKS1* mutation (target site modification)	Hot spot regions S645P and R1361H are correlated with higher MIC levels	[59]
Compensatory pathways	Calcineurin stimulates integrity signalling pathway Hsp90 regulates calcineurin function The Rho1 protein mediates a response in the cell integrity pathway Histone deacetylase Hst3 in animal models reduces fungal kidney burden Histone acetyltransferase RTT109 deletion increases susceptibility to micafungin and caspofungin	Stimulates chitin synthesis Its compromise reduces tolerance to the echinocandins Stimulates chitin synthesis through the PKC1, PKC and Sec3 pathways	[14, 72]
Biofilm formation	Multifactorial mechanism involving the overexpression of target molecules, limited diffusion, efflux pumps, tolerance and cell density		[73]

1.6.2. *FKS1* mutations and clinical breakpoints (CBP)

β–1,3-D-glucan synthase is involved in fungal cell wall synthesis, as well as being the main target for echinocandins. Genetic studies regarding caspofungin resistance in *S. cerevisiae* and *C. albicans* have indicated that the major glucan synthase Fks1 subunit is the target for echinocandins. Target-site modification was the likely cause of reduced susceptibility [59]. These findings have been confirmed in laboratory studies describing specific *FKS1* mutations associated with reduced susceptibility to echinocandins and glucan synthase activity, e.g. one specific mutation was an amino acid substitution at Ser645 in a conserved *FKS1* region (**Figure 5**) [15].

A.



B.

Hot Spot 1 (nt2628-2654, aa641-649)

Strain	DNA Sequence	Translated Sequence
Reference	ttc tcg act ttg tct tta aga gat cct	FSTLSLRDP
Baseline	ttc ttg aca ttg tct tta aga gat cct	FLTLSLRDP
Week 6	ttc ttg act ttg ttt tta aga gat cct	FLTLFLRDP
Week 36 #1	ttc ttg act ttg ttt tta aga gat cct	FLTLFLRDP
Week 36 #2	ttc ttg act ttg ttt tta aga gat cct	FLTLFLRDP

Hot Spot 2 (nt4776-4799, aa1357-1364)

Strain	DNA Sequence	Translated Sequence
Reference	gat tgg att aga cgt tat act ttg	DWIRRYTL
Baseline	gat tgg att aga cgt tat act ttg	DWIRRYTL
Week 6	gat tgg att aga cat tat act ttg	DWIRHYTL
Week 36 #1	gat tgg att aga cat tat act ttg	DWIRHYTL
Week 36 #2	gat tgg att aga cat tat act ttg	DWIRHYTL

Region sequenced: nt2517-4590 from reference (GenBank accession no. D88815)
Translated region: aa641-649, aa1357-1364

Figure 5. Hot spots and amino acid changes.

5A. Schematic diagram of *FKS1* loci associated with reduced susceptibility to caspofungin (Park *et al*., 2005). 5B. Summary of isolates' echinocandin reduced susceptibility mutations (Laverdiere *et al*., 2006).

Further studies have mapped two distinct HS regions in highly conserved areas of Fks1 which are important for echinocandin resistance. The first HS region (HS1) includes amino acids 641 to 648 in *C. albicans*, with the most prevalent substitution is located at position 645 where serine is replaced by proline, phenylalanine or tyrosine. This mutation correlates with

significantly higher echinocandin resistance levels [15]. This mutation is dominant and displays phenotypic resistance in the presence of both homozygous and heterozygous forms in *C. albicans*. Moreover, this mutation is associated with cross resistance to micafungin and anidulafungin, suggesting that the *FKS1* modification mechanism is common for the class of echinocandin drugs [59]. The second HS region (HS2) in Fks1 is located between positions 1,345 and 1,365, but mutations in this region are very low. The most prevalent substitution in clinical isolates has been Arg1, 361 to His1, 361 [74, 75]. The different mutations mentioned above show high MIC values for caspofungin in *C. albicans* isolates, (**Table 2** summarises laboratory strains and **Table 3** clinical strains).

Table 2. Distribution of *FKS1* mutations amongst *C. albicans* laboratory strains, MICs of caspofungin determined by the CLSI reference method

Laboratory strain	***FKS1* Mutation**	**Mutation nature**	**Amino acid change**	**CSF MIC values µg/mL**	**Reference**
NR2			S645/P645	>32(µg/mL)	[15]
NR4			P645/S645	>32(µg/mL)	[15]
NR3			Y645/Y645	>32(µg/mL)	[15]
T25			Δ[b]/P645	>32(µg/mL)	[15]
M70	T1922C	Homozygous	F641S	>16(µg/mL)	[76]
SC5314 (57%) M70 (50%)[a]	T1933C	Heterozygous	S645P	>16(µg/mL)	[76]
SC5314 (14%) M70 (6%)[a]	T1933C	Homozygous	S645P	>16(µg/mL)	[76]
M70	C1934A	Heterozygous	S645Y	>16(µg/mL)	[76]
SC5314 (26%) M70 (14%)[a]	C1934A	Homozygous	S645Y	>16(µg/mL)	[76]
M70	C1934T	Heterozygous	S645F	>16(µg/mL)	[76]
SC5314 (3%) M70 (6%)	C1934T	Homozygous	S645F	>16(µg/mL)	[76]
M70	G1932T C1934G	Homozygous	L644F S645C	>16(µg/mL)	[76]
A15	T1933Y		S645S/P	4(µg/mL)	[75]
A15-10	T1933C		S645P	8(µg/mL)	[75]

[a] Percentage regarding 35 mutants derived from the SC5314 strain and 50 mutants derived from the M70 strain.
[b]Δ Indicates a disrupted Cafks1 allele.

Table 3. *FKS1* mutation distribution amongst *C. albicans* clinical strains; caspofungin MICs determined by the CLSI reference method

Clinical strain	Mutation in *FKS1*	Amino acid change	CSF MIC values	Reference
CLY16996		S645F	>8(µg/mL)	[15]
CLY16997		S645P	>8(µg/mL)	[15]
CLY19230		S645F	>4(µg/mL)	[15]
CLY19231		S645F	>4(µg/mL)	[15]
W6	nt2628-2654 C-T	S645F	2(mg/L)	[74]
W6	nt4776-4799 G-A	R1361H	2(mg/L)	[74]
W36#1	nt2628-2654 C-T	S645F	2(mg/L)	[74]
W36#1	nt4776-4799 G-A	R1361H	2(mg/L)	[74]
W36#2	nt2628-2654 C-T	S645F	1(mg/L)	[74]
W36#2	nt4776-4799 G-A	R1361H	1(mg/L)	[74]
	ND	S645P	ND	[77]
Calb20464		F641Y	0.5(µg/mL) MIC80	[78]
		F641S	2(mg/L) EUCAST	[79]
CLY16996		S645F	>8(µg/mL)	[59]
CLY16997		S645P	>8(µg/mL)	[59]
M85		S645F	4(µg/mL)	[59]
M86		S645P	16(µg/mL)	[59]
M89		S645Y	16(µg/mL)	[59]
NR3		S645Y	16(µg/mL)	[59]
M195		S645F	4(µg/mL)	[59]
M196		S645F	4(µg/mL)	[59]
C31		S645P	16(µg/mL)	[59]
C41		S645P	16(µg/mL)	[59]
119	T1921C	F641L	2(µg/mL)	[75]
177	T1922C	F641S	4(µg/mL)	[75]
2762	T1922C	F641S	3.33(µg/mL)	[75]
205	T1933C	S645P	8(µg/mL)	[75]
5415	T1933C	S645P	8(µg/mL)	[75]
89	C1934A	S645Y	8(µg/mL)	[75]
85	C1934T	S645F	4(µg/mL)	[75]
149	G1942T	D648Y	2.67(µg/mL)	[75]
122	C1946A	P649H	4(µg/mL)	[75]
121	G4082A	R1361H	2(µg/mL)	[75]
194	C1934T	S645F	4(µg/mL)	[75]
194	G4082R	R1361R/H	4(µg/mL)	[75]
90	G4082R	R1361R/H	1(µg/mL)	[75]

A correlation between treatment failure *in vivo* and increased MIC for caspofungin *in vitro* has been described in clinical isolates (see **Table 2**). However, no correlation between MICs

and clinical outcomes has been established; the term "reduced susceptibility" is therefore more appropriate than "resistant" when high MICs are found [15].

A study integrating molecular, clinical and microbiological data has included an evaluation of clinical breakpoint (CBP) values in connection with reports of echinocandin resistance between *Candida* spp. [80]. The CLSI committee's 2007 definition concerning CBP MIC was that being susceptible (S) involves ≤2μg/mL for all three echinocandins and all Candida species. However, it has become apparent that clinically-resistant Candida infections involving strains having mutations in *FKS1* do not necessarily have MICs above this CBP [74, 75, 78]. Kinetic studies of the glucan synthase (GS) complex have suggested that a lower 0.25–0.5 μg/mL MIC CBP may be more sensitive in detecting strains having mutations in *FKS1*. The authors of this study thus proposed considering the CBP for susceptible (S) as being ≤0.25μg/mL, 0.5 μg/mL intermediate (I) and ≥1 μg/mL resistant (R) [80, 81].

1.6.3. Cell wall stability and drug resistance

The cell wall is a dynamic structure of which the status is related to cell physiology. Several functions have been attributed to this structure, such as being the first to come into contact with host cells, carrying important antigenic determinants of the fungus, being responsible for pathogen adherence and establishing a cross-talk with the host by the "glycan code", which includes modifications in chemical composition and cell wall polysaccharide linkages. The expected outcomes of these interactions are either the development of a pathogenic state or an immune host response [32, 82].

The *Candida albicans* wall is dynamic because it switches morphology in response to the environment and stress. It is composed of three major polysaccharides: β-1-3 glucan, β-1-6 glucan (branched glucose residue polymers containing β-1-3 and β-1-6 linkages) and chitin (N-acetyl-D glucosamine (GLcNAc) unbranched polymers containing β-1-4 bonds) [32, 83] (**Figure 6**). Cell wall proteins (CWP) play key roles like participating in cell wall assembly, helping in adhesion to host surfaces, acting as immunomodulator and protecting the fungus from host enzymes. These factors contribute directly to *Candida albicans* virulence [83].

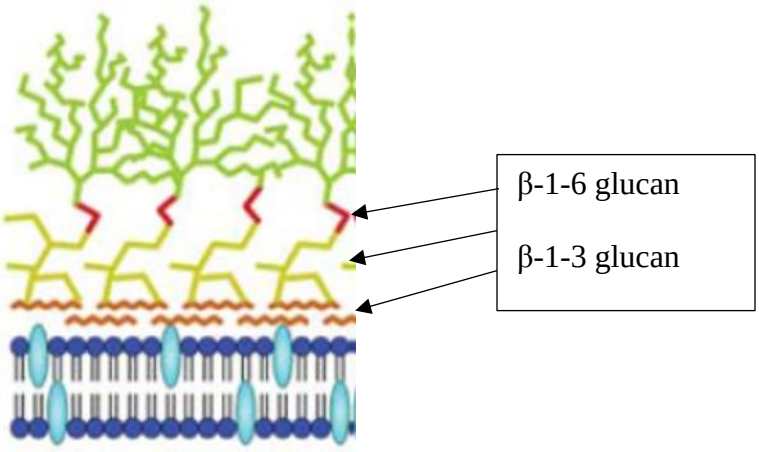


Figure 6. Cell wall composition (adapted from Cowen and Steinbach, 2008)

Fungal cell wall integrity is essential for these pathogens' survival within a changing environment. Several therapeutic targets have thus been identified, amongst which echinocandins, which act as non-competitive inhibitors of β-1-3-D-glucan synthase responsible for producing β-1-3-D-glucan, the major *Candida albicans* cell wall biopolymer, are of particular importance. Altered β-1-3-D-glucan synthesis causes severe cellular stress leading to fungal death (fungicidal activity) (**Figure 7**). Fungi having mutations in *FKS1* have an echinocandin-resistant phenotype (**Figure 8A**) which avoids cell wall damage.

Additionally, reduced susceptibility to echinocandins is associated with cell wall biosynthesis mechanisms and the preservation of its integrity as fungal responses on exposure to this type of drug [14, 59].

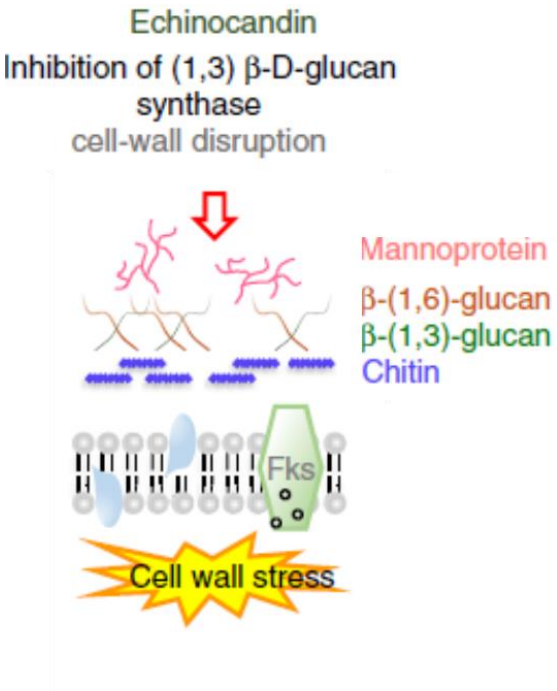


Figure 7. Echinocandin activity (adapted from Martinez *et al*., 2014)

β-1-3-D-glucan synthase is an enzyme complex having a minimum of two subunits: Fks1 and Rho; Fks1 seems to be the catalytic subunit. Rho, a GTP-binding protein from the Rho/Rac subfamily of Ras-like GTPases, facilitates glucan synthase activity. Echinocandin drugs induce stress on the cell wall by shutting down glucan synthase, resulting in glucan depletion. Some authors have proposed that Rho 1 also leads to a range of secondary interactions including the activation of the PKC cell integrity pathways by upregulating the synthesis of other cell wall components, such as chitin, through Pkc1 (**Figure 8A**) [59, 84]. Some laboratory results have shown greater polysaccharide content in the cell wall of

resistant strains [85]; other reports have shown that susceptibility can be correlated with chitin level and cell wall thickness. Caspofungin sensitive strains, for instance, have a low chitin level and thin cell wall in more than half the cases when these strains were tested against this antifungal agent [36].

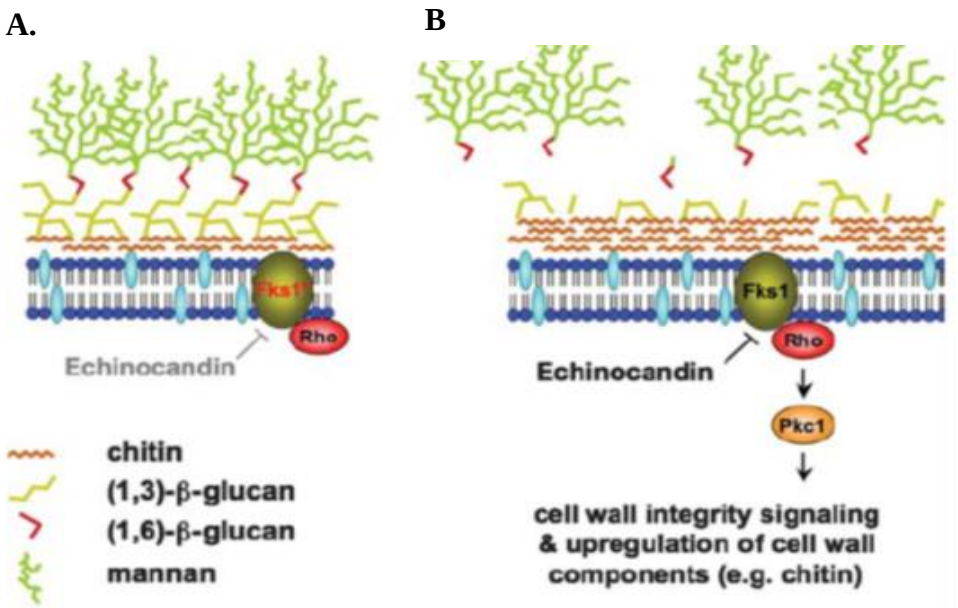


Figure 8. Mechanisms regarding response against echinocandin activity (adapted from Cowen *et al*., 2008).

Novel compensatory pathways are described through functional studies involving cell wall stress conditions; one of them describes the activity of genes such as *SKO1* and *MKC1*. These genes form a component in the response to osmotic stress in the HOG pathway and its activity changes in the presence of caspofungin as a factor causing cell wall stress [37, 86, 87]. *MKC1* plays an important role related to activity in the PKC pathway; an interesting study has shown this gene's interaction with the calcineurin pathway where it has inhibited *MKC1* expression when this pathway has been inhibited by cyclosporine (calcineurin pathway inhibitor) and

susceptibility to the caspofungin agent has increased [86]. Regarding the calcineurin pathway, evidence has revealed virulence related to the presence of *CNB1* in an experimental murine model infected with mutant *cnb1/cnb1* strains where these genes were avirulent. Furthermore, there was increased susceptibility to fluconazole and micafungin was fungicidal in the null mutant [72, 86, 88].

Additional studies in microarray assays with strains tested with caspofungin have revealed more genes involved in the compensatory response to preserve cell wall integrity (CWI) and shown that the compensatory pathway leads to the mitogen-activated protein kinase (MAPK) pathway. *CRH11, ERG5* and *PNG62* genes were checked regarding their expression when exposed to caspofungin and may play a significant role [37, 38].

The aforementioned molecular mechanisms form part of regulatory circuitry governing emerging drug resistance against echinocandins where patient treatment and medical staff behaviour/approach (such as using prophylaxis) can increase the risk of inducing resistance regarding this pathogen [89]. Further research is required for a better understanding of these compensatory pathways because they represent a new field related to stress, evolution and antifungal drug resistance [84].

1.6.4. Drug resistance and paradoxical effect

Drug resistance and paradoxical effect refers to the growth of caspofungin (one of the echinocandins) susceptible *Candida albicans* after 24 hours at concentrations far exceeding the MIC. This effect has been observed *in vitro* with strains having a normal susceptibility

pattern. Paradoxical growth has not been related to *FKS1* mutations, does not involve the gene encoding glucan synthase, nor upregulation of β-1-3 glucan synthesis [59, 90].

The mechanism related to this effect is still unclear; however, some authors have proposed that the gene has been promptly inactivated by a high caspofungin concentration; however, a compensatory upregulation of another cell wall component's synthesis is then started. Study developed by Stevens *et al.*, 2006 observed that in presence of echinocandin, chitin synthesis act more synergically *in vitro* and that if one cell wall polymer is inhibited then the other might compensate; however, the fungus becomes exposed if both are blocked. This research showed that β-1-3-glucan in cell wall content decreased by 81% and β-1-6-glucan by 73%, whilst chitin increased by 898% in the presence of caspofungin [91].

Other interesting results have suggested that generalised compensatory responses to a range of cell wall injuries were displayed during the paradoxical effect, including Hog1 activation (high-osmolarity glycerol response) and calcineurin stress response pathways, the latter leading to an increase in chitin synthesis. However, blocking the calcineurin pathway with tacrolimus (FK506) or cyclosporine A and/or inhibiting chitin synthesis with nikkomycin Z eliminated paradoxical growth [90].

1.6.5. Virulence factors and resistance

Microorganisms' virulence factors are essential for causing disease, being directly related to their pathogenicity. The association between echinocandin-related virulence and resistance must be studied for a better understanding of host-pathogen interactions.

C. albicans has many virulence factors, such as the ability to grow at 37°C, hypha transformation, cell adhesion and biofilm formation. The following should be stressed:

- **Proteases**: Cell surface-associated proteases (Saps) are hydrolytic enzymes and major virulence factors for *C. albicans*; they are encoded by a 10-member gene family (Sap1-Sap10). Saps can be attached to the cell membrane, incorporated into the cell wall via glycosylphosphatidylinositol (GPI) anchors (Sap9, Sap10) or released into the extracellular space. These proteases' main roles are to provide nutrition for cells and aid penetration and evasion of the immune system [92]. Previous work studying GPI-anchored Saps and their role in cell wall modification on echinocandin exposure has shown that Sap9 and Sap10 activity is required for cell surface stability and *SAP9* gene expression is increased when infection by *Candida albicans* receives antifungal treatment with fluconazole and caspofungin [36, 46, 92];
- **Other GPI-anchored proteins** which become altered upon echinocandin exposure and which affect susceptibility are encoded by *SSR1, MID1* and *PGA31* which, when deleted, have increased susceptibility to caspofungin, whilst when *DFG5, PHR1*, *PGA4* and *PGA62* were deleted they decreased susceptibility to caspofungin [36]. The main genes involved in invasion and adhesion are *HWP1, EFG1* and *ALS3* [93-95]; and
- **Calcineurin:** The calcium-regulated signalling enzyme seems to be essential for *Candida albicans* virulence, especially for survival in serum. Inhibiting this enzyme has a synergistic effect with echinocandins [14].

An interesting study regarding resistance and virulence was published in 2008. Its main conclusions were that (1) antifungal drug resistance increased *Candida albicans* virulence for systemic infections, (2) the drug action and (3) resistance mechanisms were independent of increased virulence and acquired resistance to stress might induce biological compensatory changes conferring advantages on pathogenicity and virulence so the worst threat is related with emerging resistance in clinical setting that increases clinical treatment failure [55, 85].

Although several fungal virulence factors have already been described, many more are yet to be discovered. The discovery of further virulence factors and their relationship with resistance will allow a better understanding of the main mechanisms involved in emerging nosocomial infectious diseases and identifying their potential harm for establishing preventive measures in order to minimise this problem.

1.7. MIC assay and echinocandins

The Clinical Laboratory Standards Institute (CLSI) and the European Committee for Antimicrobial Susceptibility Testing (EUCAST) have developed guidelines for broth microdilution for susceptibility tests for yeasts [96, 97]. Despite both methods have methodological and interpretative breakpoint differences, they are equivalent. Other methods are commercially available, such as E-Test, Sensititre and Vitek.

The aforementioned methods identify fungal strain susceptibility and pathogen growth is measured within a series of drug concentrations over a defined period of time according to

standard protocols. MIC is defined as the lowest concentration of an antifungal drug which inhibits growth by either 50% or 90%. Analysing and interpreting these results shows how a pathogen will respond to antifungal treatment; however, comparing *in vivo* outcomes to *in vitro* ones does not necessarily match, since many factors are involved, such as pharmacokinetics, host-pathogen interactions, microbial interactions and environmental factors. Fungal species often have differing intrinsic levels of drug tolerance and specific mechanisms of resistance may be acquired. Even in the absence of specific resistance mechanisms, species and strains differ regarding their ability to survive and proliferate during drug exposure, regardless of changes in MIC (often referred to as drug tolerance). Tolerance can enable the evolution of drug resistance, allowing a population of proliferating cells to respond to selection imposed by a particular drug [14]. Micafungin or anidulafungin use has recently been proposed instead of caspofungin in susceptibility tests because of variability regarding the break-points obtained by several laboratories [98]. According to this guideline, informational supplement M27-S4/2012 must be followed [99].

1.8.Clinical Break Points for echinocandins

The interpretive break point for anidulafungin, caspofungin and micafungin, according to the CLSI M23-A2 guidelines is ≤2μg/mL for 98.8% to 100% of all *Candida* spp. clinical isolates [100]. Several studies have been aimed at ascertaining echinocandin *in vitro* activity. **Table 4** and **Table 5** summarise the *in vitro* antifungal data related to echinocandins against *Candida albicans* with corresponding references.

Table 4. *In vitro* echinocandin activity

Fungus	Isolate #	Year	AF agent	MIC range (µg/mL)	MIC90 (MIC50 or [a]G µg/mL)	MIC 50 µg/mL	MIC 90 µg/mL	MFC range (µg/mL)	Reference
C. albicans	2394	2003	Anidulafungin	<0.01->8	0.01-0.05			0.03-4	[101]
	4265		Caspofungin	<0.01->8	0.12-1.0			0.12>8	[101]
	966		Micafungin	<0.01-0.5	0.01-0.25			0.06-2	[101]
C. albicans	733	2003	Anidulafungin			0.03	0.03		[102]
			Caspofungin			0.5	0.5		[102]
			Micafungin			0.03	0.03		[102]
C. albicans	1483	2007	Anidulafungin			0.03	0.12		[6]
			Caspofungin			0.03	0.06		[6]
			Micafungin			0.015	0.03		[6]
C. albicans	143	2010	Anidulafungin	<0.016-1		0.016	0.25		[103]
C. albicans	32	2010	Anidulafungin	<0.008-0.12					[104]
			Caspofungin	<0.008-1					[104]
			Micafungin	<0.008-0.25					[104]
C. albicans	4283	2010	Anidulafungin	0.007-1					[105]
			Caspofungin	0.007-0.5					[105]
	4282	2010	Micafungin	0.007-0.5					[105]

[a]G: geometric mean

Studies have shown *in vitro* susceptibility to echinocandins regarding a wide range of strains being susceptible to caspofungin at 0.06µg/mL; however, these values only concerned *in vitro* outcomes and the relationship between clinical outcomes and *in vitro* outcomes was

neglected. Several reports have dealt with resistant isolates from patients treated with these antifungals, despite their recent introduction into clinical practice [79].

According to epidemiological surveillance studies carried out around the world, excellent levels of *in vitro* echinocandin activity have been observed during a six year follow-up, suggesting that emergent resistance to echinocandins is hardly likely since the caspofungin inhibition percentage for *C. albicans* was 99.6% [10]. However, later studies have shown the need for re-evaluating the break points defining echinocandin susceptibility as clinical observations have been made regarding therapeutic failure, in spite of susceptibility tests having indicated lower than 2μg/mL values. Such break-points were adjusted for defining susceptibility as ≤0.25μg/mL, intermediate susceptibility as 0.5 μg/mL and ≥1 μg/mL for resistance [80]. A recent review of clinical break-points has proposed that just ≤0.25μg/mL should be used for susceptibility and ≥0.5 μg/mL for resistance [106].

Table 5. Echinocandin *in vitro* susceptibility

Organism	AF agent	No. of isolates tested	Cumulative % of isolates susceptible at MIC (µg/mL):										Reference
			0.007	**0.015**	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4 >8**	
C. albicans	**Caspofungin**	1.476	2	28	68	**98**	>99	>99	100				[107]
	Micafungin	1.476	10	76	**95**	>99	>99	100					[107]
C. albicans	**Anidulafungin**	2.869	6.2	33.5	69.5	**92.4**	99.1	99.5	99.5	99.6	100		[10]
	Caspofungin	2.869	1.7	26.7	74.2	**97.1**	99.3	99.9	100				[10]
	Micafungin	2.869	11.9	80.6	**96.4**	99.3	99.4	99.5	99.6	100			[10]
***C. albicans*[a]**	**Anidulafungin**	533	6.4	31.5	66.2	**91.4**	99.2	99.6			100.0		[10]
	Caspofungin	533	1.3	23.6	70.4	**95.5**	99.1	100.0					[10]
	Micafungin	533	10.7	78.8	**95.3**	99.6				100.0			[10]
C. albicans	**Anidulafungin**	10		10	50	80	100						[108]
	Caspofungin	10			10	70	100						[108]
	Micafungin	10				70	100						[108]
C. albicans	**Anidulafungin**	4.283	7.89	37.73	73.73	**94.65**	99.70	99.98		100.00			[105]
	Caspofungin	4.283	2.15	29.72	77.28	**98.25**	99.84	99.98	100.00				[105]
	Micafungin	4.282	14.20	83.14	**97.73**	99.84	99.95	99.98	100.00				[105]

1.9. The aims of this work

This work has been aimed at identifying the main factors related to the dual role of *FKS1* on virulence and resistance in clinical nosocomial *Candida albicans* isolates from ten hospitals in Colombia, regarding the relevance in the therapeutic approach for echinocandins the latest antifungal group, for treatment the infectious diseases related with these fungi. These isolates were characterised for the drug susceptibility pattern of a set of *Candida albicans* nosocomial strains in response to echinocandins, as new molecules in the Colombian hospitals. These nosocomial strains' phenotypical traits involved in virulence and resistance were then described and the association between clinical risk factors, phenotypic traits and the presence of reduced susceptibility to echinocandins in nosocomial strains of *C. albicans* was established as a strategy related with a better knowledge in the epidemiology of nosocomial infection by these fungi.

Chapter II - Global approach and methodology

To achieve this work's aims and taking into account the work's clinical purpose, an approach was based on the main phenotypic characteristics identified to date, encompassing the set of isolates' molecular expression. A descriptive observational study was thus designed, where sample size was taken by convenience. Selected clinical isolates which had been identified as nosocomial *C. albicans* were collected during a 7-month period from ten tertiary-care centres' total discharges.

1.1. Ethical aspects

This research's main purpose was to contribute towards understanding virulence-related mechanisms and *C. albicans* nosocomial infection resistance, to improve preventative, diagnostic and therapeutic interventions. The nosocomial strains were collected in Bogotá (Colombia) following previous approval by the Ethics Committees from the ten health institutions included in this study. According to Colombian law, the study of clinical samples with microorganisms is considered to involve minimum risk [109] related to confidentiality and privacy protection of patient's information. Collected samples were irreversibly anonymised to protect patients' identity. Research team members disclosed no potential conflicts of interest [110].

1.2. Clinical strains

The clinical isolates were taken after analysing 82,957 hospital discharges as reported by ten tertiary care complexity institutions from November 2007 to May 2008 (i.e. 44% of Bogota's tertiary care hospitals). A search was made for healthcare-associated infection (HAI) diagnoses when analysing the hospital discharges; these were differentiated into two groups for such analysis: the first (5,204) consisted of people discharged by these institutions' general ICUs and the second (77,763) referred to these institutions' hospitalisation floors.

All discharged patients' data was analysed, together with each participating institution's infection committee's data; HAI-related clinical histories were identified according to the pertinent medical literature [51, 111]. Altogether, 101 infections caused by *Candida spp*. were obtained; 40 clinical isolates were identified by conventional methods as *Candida albicans* (Figure 9).

A second sample-collecting phase occurred during 2014; 240 additional isolates were obtained from a tertiary care hospital; they were initially identified as *C. albicans* by conventional MALDI TOF MS methodology; these clinical isolates did not come from nosocomial infections and were used exclusively for identifying additional atypical Candidas by MALDI TOF MS.

As well as the 40 nosocomial *C. albicans* obtained during phase 1, 6 additional isolates were obtained during phase 2. **Table 6** gives general information concerning the isolates' origin and main clinical features.

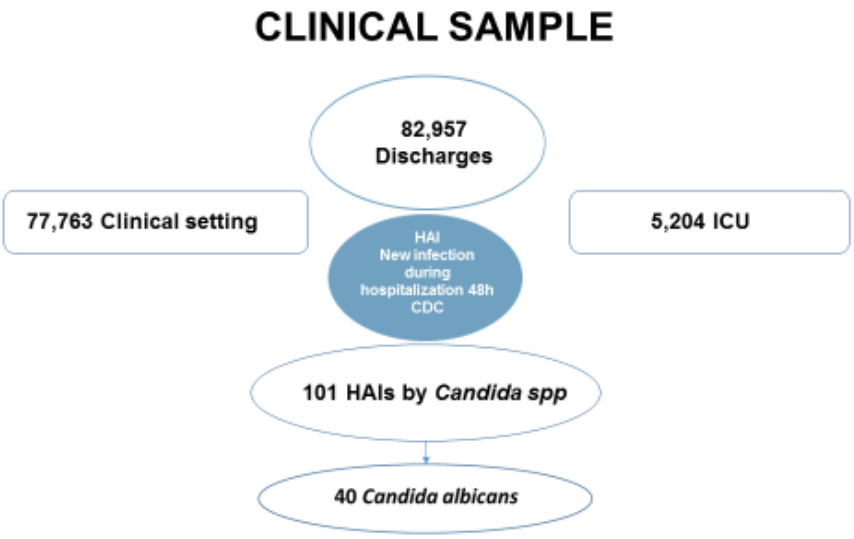
Table 6. Clinical isolates

Isolate	Conventional Species ID	Patient Gender	Age (Years)	Source	Clinical setting	Hospital locality
UR5	*Candida albicans*	F	72	Urine	ICU	Kennedy
UR6	*Candida albicans*	F	55	Urine	ICU	Kennedy
UR8	*Candida albicans*	F	58	Urine	ICU	Kennedy
UR10	*Candida albicans*	M	80	Blood	ICU	Kennedy
UR29	*Candida albicans*	F	68	Urine	Room	Kennedy
UR41	*Candida albicans*	M	1 month	Blood	ICU	Usaquén
UR48	*Candida albicans*	F	5 month	Blood	Room	Usaquén
UR78	*Candida albicans*	M	41	Urine	ICU	Kennedy
UR107	*Candida albicans*	F	1 month	Blood	ICU	Tunjuelito
UR111	*Candida albicans*	M	18	Urine	Room	Tunjuelito
UR115	*Candida albicans*	M	49	Urine	ICU	Tunjuelito
UR118	*Candida albicans*	F	31	Blood	ICU	Usaquén
UR122	*Candida albicans*	M	1 month	Urine	ICU	Usaquén
UR143	*Candida albicans*	F	80	Blood	ICU	Usaquén
UR144	*Candida albicans*	F	65	Blood	ICU	Usaquén
UR147	*Candida albicans*	F	50	Blood	ICU	Usaquén
UR213	*Candida albicans*	F	1 month	Urine	ICU	Mártires
UR219	*Candida albicans*	F	5	Blood	Room	Usaquén
UR221	*Candida albicans*	M	1 month	Blood	ICU	Usaquén
UR230	*Candida albicans*	F	1 month	Blood	ICU	Kennedy
UR242	*Candida albicans*	M	6	Urine	ICU	Mártires
UR257	*Candida albicans*	F	75	Urine	ICU	Tunjuelito
UR263	*Candida albicans*	M	2	Blood	Room	Teusaquillo
UR282	*Candida albicans*	F	70	Urine	Room	San Cristobal
UR317	*Candida albicans*	F	64	Urine	Room	Tunjuelito
UR385	*Candida albicans*	M	2	Blood	ICU	Usaquén
UR388	*Candida albicans*	F	47	Urine	ICU	Usaquén
UR390	*Candida albicans*	M	56	P.F	ICU	Usaquén
UR393	*Candida albicans*	M	17	P.F	ICU	Usaquén
UR404	*Candida albicans*	M	4 months	Urine	ICU	Usaquén
UR407	*Candida albicans*	M	57	Blood	Room	Usaquén
UR408	*Candida albicans*	M	5 months	Urine	ICU	Usaquén
UR424	*Candida albicans*	F	44	Urine	ICU	Kennedy
UR425	*Candida albicans*	M	70	Urine	ICU	Kennedy
UR426	*Candida albicans*	F	2	Urine	ICU	Mártires
UR475	*Candida albicans*	F	4	Urine	ICU	Mártires
UR481	*Candida albicans*	M	48	Blood	Room	San Cristobal
UR527	*Candida albicans*	M	17	Blood	ICU	Tunjuelito
UR600	*Candida albicans*	M	1 month	Blood	ICU	Usaquén

UR608	*Candida albicans*	F	84	Urine	ICU	Usaquén
EZ41	*Candida albicans*	M	44	BAL	Room	Chapinero
EZ44	*Candida albicans*	F	26	Vaginal swab	N.D	Chapinero
EZ60	*Candida albicans*	M	94	Urine	Room	Chapinero
EZ69	*Candida albicans*	F	68	Faeces	Room	Chapinero
EZ77	*Candida albicans*	M	18	BAL	Room	Chapinero
EZ80	*Candida albicans*	F	73	BAL	Room	Chapinero

Note: ICU: Intensive care unit, P.F: Peritoneal fluid, BAL: Bronchoalveolar lavage, N.D: No data.

Figure 9. Clinical samples obtained from tertiary care hospitals in Bogotá, Colombia.



1.2.1. Reference strains

A set of reference strains was used in the assays. Firstly, SC5314, a clinical reference strain, was used in the susceptibility assays (MIC test) along with *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 quality control strains [112]. SC5314, *C. albicans* ATCC 90028,

C. dubliniensis ATCC MYA-646 [25], *C. africana* ATCC 2669 [21] and *C. glabrata* ATCC 2001 were used for assays related to phenotypic characterisation [113]. **Table 7** gives general information regarding these reference strains.

Table 7. Reference strains

Strain	Genotype	Reference
SC5314	Wild type	(Gillum *et al*., 1984)
***C. albicans* ATCC 90028**	Wild type	(Pfaller *et al*., 1994)
***C. krusei* ATCC 6258**	Wild type	(Pfaller *et al*., 1994)
***C. parapsilosis* ATCC 22019**	Wild type	(Pfaller *et al*., 1994)
***C. dubliniensis* ATCC MYA-646**	Wild type	(Sullivan *et al*.,1995)
***C. glabrata* ATCC 2001**	Wild type	(Koszul *et al*., 2003)
***C. africana* ATCC 2669**	Wild type	(Tietz *et al*., 2001)

1.3. Characterising the susceptibility or resistant pattern of a set of nosocomial strains

1.3.1. MIC screening test for reference and clinical strains

Susceptibility to antifungal agents was measured in liquid broth microdilution assays. The method outlined in CLSI document M27-A3 was used for determining antifungal MIC for all clinical *C. albicans* isolates. An additional set of assays was carried out, as outlined in the latest version of CLSI document M27-S4 [96, 99]. The initial assays were carried out in the KULeuven's MCB laboratory (Belgium) and additional assays in the CIB reference laboratory (Medellín, Colombia) for the M27-S4 version. All assays were done in triplicate and repeated thrice to guarantee test reproducibility.

Only six nosocomial *Candida albicans* isolates were used for the additional set of assays with the M27-S4 version, giving caspofungin resistance break-points with M27-A3 methodology and also having faster growth than that for the SC5314 clinical reference strain.

1.3.2. Quality control

Quality was controlled according to CLSI indications, using those recommended for the *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 strains [96]. Quality control strains were included for each susceptibility assay to verify which MIC break points agreed with those described in the CLSI M27-A3 guidelines. The assays were done in triplicate with three repeats at different times to guarantee test reproducibility.

1.3.3. Antifungal drugs

Caspofungin (Merck), micafungin (Astellas) and fluconazole (Sigma) were used in this study. The working solution for each antifungal drug was prepared in sterile water following CLSI M27–A3 guidelines; the first two dilutions were in water and final dilutions in RPMI 1640 solution adjusted to pH 7.0 with 0.165M morpholinepropanesulfonic acid (MOPS) buffer [96]. *C. albicans* nosocomial isolates were tested with micafungin dissolved in DMSO, following CLSI M27-S4 recommendations [99].

1.3.4. Searching for mutations in echinocandin-resistant strains

The *FKS1* gene was sequenced to identify mutations, aimed at finding putative mutations in HS1 and/or HS2. Previous studies have reported point mutations for HS1 between amino

acids 640 to 650 (serine replaced by proline, tyrosine or phenylalanine) and for HS2 between amino acids 1345 to 1365 [15].

FKS1 gene sequences were amplified using previously described primers for HS1 and HS2 regions: F 5' GTTCCACCAGTTTATACATTCC 3' and 5' ATGTCACTCTTGAGAATTGATC 3' R for HS1 and F 5' GCTCATGAAGCTATCATGTGTT 3' and 5' CAAGACAAACACCTAAACATCC 3' R for HS2 [15, 74]. All reactions involved using KAPA HiFi containing 0.3 µM of each primer in a final 25µL volume. Thermal conditions were set as follows: denaturing at 95°C for 5 min, followed by 35 cycles of denaturing at 98°C for 30 seconds, primer annealing at 54°C for 20 seconds and an extension step at 72°C for 1 min followed by a final extension step at 72 C for 5 min. Attempts were made to characterise mutations on at least two different occasions, forming part of two subsequent biological repeats; the amplification products were purified by using a Promega Wizard purification systems kit and then sent for sequencing in both directions with a BigDye Terminator kit (Macrogen, Seoul, South Korea).

1.4. Phenotyping of clinical strains

1.4.1. Cell adhesion and biofilm formation

Biofilm growth on the surface of wells from pre-sterilised, polystyrene, flat-bottomed, 96-well microtitre plates was determined (Corning Incorporated, USA) **[114]**. Cell viability or cell count was measured at different stages of biofilm formation, including the adhesion phase (90 minutes) and mature biofilm stage (24h).

1.4.2. Growth and morphology assays

Growth curves were determined with a Bioscreen apparatus (Life Sciences). Planktonic cell growth was determined at 30°C in RPMI media for 12 hours **[115]**. Specific hypha-inducing solid media, such as Spider medium, was used for hypha induction. Plates were incubated at 37°C and colony formation was observed for 7 days **[116]**.

1.4.3. Culture medium for atypical isolates

All isolates were cultured using identical conditions. The samples were inoculated into Sabouraud agar (Difco, St Louis, Mo) for 24–48 hours at 30°C, 37°C, 42°C and 45°C. Identity was confirmed by conventional identification methods, such as germ tube induction, microscopic morphology and chlamydospore formation in corn meal agar (Oxoid, Basingstoke, United Kingdom) [117]. The SC5314 strain was used as a positive control for chlamydospore formation and the *Candida glabrata* ATCC 2001 strain as negative control. Photographs were taken by with an optical light microscope (Leica icc50HD) at 40x.

Samples were also plated on CHROMagar (Becton Dickson, Meylan, France) to verify the clinical isolates' chromogenic presentation, read after 48 hours incubation, according to the manufacturer's recommendations. The carbohydrate assimilation pattern was evaluated in standardised serial assays on yeast nitrogen base (YNB) agar, containing trehalose, glucosamine and then sucrose as sole carbon source (5g/L). The amount of yeast inoculated into the medium was 10^3 CFU; this was read after 2 and 5 days incubation at 30°C [21].

1.4.4. Identification using API 20C AUX for atypical isolates

Fresh cells were collected after culturing in Sabouraud for 48 hours at 30°C. An API 20 C AUX (bioMériux, France) kit was used, according to the manufacturer's instructions. The data regarding the results was collected 48 hours later and the growth in each cupule was read manually. The codes obtained were analysed using APIweb software (bioMériux, France).

1.5. Gene expression

Real-time PCR was used for characterising the transcriptional expression of some virulence factors to establish the relationship between virulence and resistance **[94]**.

Cultures were diluted to OD_{600} at 0.2 in yeast extract-peptone-dextrose (YPD) and divided into two separate sets for incubation at 37ºC; the first batch was grown in the presence of caspofungin at MIC50 concentration (0.125µg/mL) with RPMI for 24 hours whilst the second batch was grown without caspofungin but with RPMI for the same time. RNA was extracted by using TRIzol RNA isolation reagent (Life Technologies). RNA concentration was measured by NaNoDrop spectrophotometer (ND 1000 Life science). Isolated RNA was subjected to DNase, RNase free treatment and adjusted to 1µg/µL final concentration. An iScript cDNA synthesis kit (BIO-RAD) was used for obtaining cDNA. Real time PCR was performed in 96-well plates using the Step One Plus Real Time System (Applied Biosystems) and GoTaq qPCR Master Mix (PROMEGA); 5µL of 0.4 ng/µL and 15µL Mastermix (containing the primers) was added to each reaction involving standardised cDNA sample concentration. The real time PCR reactions were performed at 95ºC for 2 minutes, followed

by 40 cycles (denaturation-annealing/extension) at 95ºC for 3 seconds and 60ºC for 30 seconds. The expression of control gene *ACT1* was used during the assay and normalised data were then used for calculating relative gene expression levels. The primers were analysed for particular *C. albicans* genes before starting standard curves with gDNA; the assays were performed in three biological repeats. The data for each target gene was calculated as fold change compared to the *ACT1* reference gene using the ΔΔCt quantification method [118]. **Table 8** lists the sequencing primers.

Table 8. Primers used for Quantitative Real Time – PCR

Primer		**Primer Sequence (5' ⟶3')**	**Origin**
ACT 1	fwd	TTTCATCTTCTGTATCAGAGGAACTTATTT	This study
	rev	ATGGGATGAATCATCAAACAAGAG	This study
TEF 1	fwd	CCACTGAAGTCAAGTCCGTTGA	This study
	rev	CACCTTCAGCCAATTGTTCGT	This study
GSC1	fwd	GTTTCAATTTTGCCAGATTAA	This study
	rev	AACATAAATCATGGTGGCAAT	This study
CNB1	fwd	TGAAGGACGAGGAACTACAACAAA	Van Hauwenhuyse *et al*., 2014
	rev	TTGCCATCACCGTCCAAAT	
SOD5	fwd	ATTTCAATCCTTACAATGGAA	This study
	rev	ATGAGCAGCAGGAGTTGCAGC	This study
ERG5	fwd	AGATACCGTCCACCAGTCTTG	This study
	rev	AAGGATTTCTTAACAACGTAT	This study
PGA23	fwd	ACTGGAAACTCAGTTGCTACT	This study
	rev	CCTAAACCTGCTGGACCAGAG	This study
PGA13	fwd	AGCAGCCATACTACTCAAGTG	This study
	rev	GGCACTGAGGCTGAGGAGACA	This study
PGA62	fwd	GCTGTCACCACCGGTGTCACC	This study
	rev	GTGTAGATGGTAGTACCTTCA	This study
PGA31	fwd	CTCCAGTCTACAGCAACAGTA	This study
	rev	TGGACAGTAAGTGGTGTAAAG	This study
PNG2	fwd	GTTCTTATTCAAGATAACGAC	This study
	rev	TGTTCAGTGACATCTTTAGAC	This study
MKC1	fwd	CAGTTTCCAGCTCAAGAGAGT	This study
	rev	TTATTGTAAGGGGTCTGAGAA	This study
SKO	fwd	GGTTATAGAGAATTATCTGCT	This study

	rev	AATATGGCACCACGCAATTCA	This study
GSL22	fwd	AATAGATCACAACGGAGGAAA	This study
	rev	ATAAAGTATTTTATTATACGC	This study
CRH11	fwd	TTGGAAAGTGGACAATCTGTT	This study
	rev	GATGGACTGGATGAACTATCA	This study

1.6. Fluorescence intensity in stress condition with caspofungin MIC50

Reference strain SC5314 and the clinical isolates shown having reduced susceptibility to caspofungin, were cultured at 30ºC in YPD with shaking at 200 rpm overnight [119]. The samples were diluted at OD_{600} at 0.2 in YPD and divided into two separate sets for incubation at 37ºC for six hours; the first batch was grown in YPD with shaking at 200 rpm in the presence of caspofungin at MIC50 concentration (0.125µg/mL) whilst the second batch was grown in YPD with shaking at 200 rpm, without caspofungin during the same time. The cells were stained with 25µg/mL^{-1} Calcofluor white (CFW) (Sigma-Aldrich, United Kingdom) and fixed with 0.1mg/mL concanavalin A (Sigma-Aldrich, United Kingdom) to visualise chitin. All samples were examined using a confocal laser scanning biological microscope (Olympus FV1000). Images were digitally recorded with the accompanying FV10ASW3.1 software. Mean fluorescence intensities were analysed with ImageJ software and simple random sampling and univariate analysis was used with each sample. The statistical tests used were Levene's test for homogeneity of variance and Welch and Brown Forsythe tests for equality of means. Reference strain SC5314 and clinical isolates were compared by searching for significance difference regarding fluorescence intensities.

1.7. MALDI TOF characterisation

The clinical isolates were cultured in Sabouraud agar for 24 to 48 hours at 30°C. MALDI-TOF was used for MS identification using the protein extraction in formic acid/ethanol method, according to the Bruker Daltonics' protocol, with minor modifications as reported by Cendejas-Bueno *et al*., (2012) [120]. The identification spectrum was produced from 240 laser shots in duplicate and compared to the equipment's (Bruker) mass spectrum library. At least three independent measurements were performed for each clinical isolate. The MALDI-TOF MS results were then compared and a score was obtained according to the manufacturer's technical specifications as follows: correct genus and species identification (≥2.0), secure genus identification (1.7–2.0) and no reliable identification (<1.7).

The mass spectra identified during measurements were visualised by Bruker flex analysis software and MALDI Biotyper RTC [120]. The original, commercially-available Bruker database (BDAL) is regularly updated by the manufacturer. This research was carried out with a BDAL library containing 4,110 main spectra (MSPs) created between 2007 and 2012.

1.7.1. MALDI spectra analysis for atypical isolates

Initial MALDI TOF characterisation of all clinical isolates led to a subgroup of clinical isolates being observed which was classified as *C. albicans – C. africana*; 5 of them belonged to the first group of clinical isolates obtained in 2007 -2008 (phase 1) and a further 6 isolates to the group of clinical isolates revised in 2014 (phase 2).

Twenty readings were taken per isolate. Peak readings having less than 5% relative intensity and signal/noise less than 3 were discarded. The spectra obtained for each isolate were combined (11 resulting spectra, one for each isolate) and compared to 4 reference (r) spectra from *C. albicans* (strains ATCC 90028, SC5314), *C. dubliniensis* (ATCC MYA-646) and *C. africana* (ATCC2669) obtained from the Microbiology Service at La Fe teaching hospital (Valencia, Spain).

Data binning was used for reducing the effects of minor observation errors for each spectrum, using 10 m/z as bin interval (ranging from 2,010 to 15,680). The presence of a peak in a bin was scored as 1 and its absence 0. Distance matrixes were estimated on the resulting binary matrix (19 x 376) using absolute Pearson and Euclidian measurements; a cluster analysis was then performed using UPGMA and complete methods. Unbiased bootstrap values were used to support the obtained trees (1,000 replicas), all using the R 3.1.3 *pvclust* package [121-123]. Metric multi-dimensional scaling (MDS) was also performed on each distance matrix, using the R 3.1.3 *stats* package [122].

1.8. Molecular characterisation:

The following molecular approach was taken for the eleven *Candida albicans*-related species.

1.8.1. Molecular marker amplification and sequencing

The D1/D2 region of the rRNA gene complex 28 subunit was amplified following international guidelines for the molecular identification of fungi for Candida identification [124]. The hypha cell wall protein 1 (*HWP1*) gene [125] was also used.

Genomic DNA was extracted from isolated colonies grown in Sabouraud dextrose agar (Becton Dickinson and Co.) using a QIAamp DNA mini kit (QIAGEN, Germantown, MD) following the manufacturer's recommendations. The molecular markers were amplified using the primers and protocols previously described for the D1/D2 region [124] and the *HWP1* gene [125]. The D1/D2 region amplified products (~600 bp) were sent to Macrogen (Maryland, USA) for Sanger bidirectional sequencing. Sequencher 5.0 software (Gene Code Corporation) was used for editing and aligning the sequences. A search was then made in the following databases for each obtained sequence to establish similarity with known strains: the NCBI databases (BLAST) (National Center for Biotechnology Information, Washington, DC), CBS-KNAW (Fungal Biodiversity Centre) and Mycobank database (International Mycological Association).

Typing by *HWP1* gene was based on amplified product size differences, ~ 700 bp for *C. africana*, ~ 941 bp for *C. albicans* and ~ 569 bp for *C. dubliniensis* [125]. The assays were done in triplicate; amplification product size was assessed on agarose gels. **Table 9** provides pertinent information about the primers used in these assays.

Table 9. Atypical *C. albicans* isolates' molecular marker primers

Primer	Name		Primer Sequence (5'⟶3')	Description	Origin
HWP1		fwd	GCTACCACTTCAGAATCATCATC	Molecular marker	(Romeo *et al*., 2008)
		rev	GCACCTTCAGTCGTAGAGACG	Molecular marker	(Romeo *et al*., 2008)
D1-D2	NL-1	fwd	GCATATCAATAAGCGGAGGAAAG	rRNA	(Wayne *et al*., 2008)
	NL-4	rev	GGTCCGTGTTTCAAGACGG	rRNA	(Wayne *et al*., 2008)

1.9. The statistical significance of the correlation between clinical risk factors and phenotypical trait expression

The data was processed using SPSS (version 20.0) and STATA (version 11.0) software. Absolute and relative frequency distribution was expressed as percentages for qualitative variables and measurements of central tendency, the average and mean dispersion; the range and standard deviation were used for quantitative variables. The association of susceptibility and mortality with different factors, Fisher's exact test and likelihood ratio were evaluated for qualitative variables and Shapiro Wilk's test was used for evaluating normality in normal variables, Student's T-test for average differences for independent groups having homogenous or heterogeneous variability, using Levene's test for evaluating the homogeneity of variance. The exact Mann Whitney test or Kruskal-Wallis non-parametric ANOVA tests were used for distributions which were different to normal or ordinal variables when comparing three or more groups. Multivariate analysis involved using binomial, multinomial and ordinal exact logistical regression models (hierarchical) and discrimination power was evaluated using area under the ROC curve. Statistical tests were evaluated using 5% and 10% significance.

These isolates were characterised regarding the drug susceptibility pattern of a set of *Candida albicans* nosocomial strains in response to echinocandins.

Chapter III - Clinical isolates and their echinocandin susceptibility patterns

One hundred and one nosocomial *Candida* spp. infections were identified during 7 months from 77,763 non-ICU ward discharges and 5,204 ICU discharges, giving an incidence rate of 12.2 *Candida spp*. nosocomial infection. per 10,000 discharges; distribution was 30.7% *C. albicans*, 22.8% *C. tropicalis*, 20.8% *C. parapsilosis*, 19.8% other *Candida* spp., 3% *C. krusei* and 3% *C. glabrata*. The distribution of nosocomial *C. albicans* infection showed that 7 clinical isolates (13%) came from non-ICU ward discharges and 24 clinical isolates (87%) from ICUs.

2.1. Antifungal susceptibility

Initial screening of the 35 clinical samples initially classified as *C. albicans* using CLSI M27-A3 methodology, revealed that 23% of the samples had a caspofungin resistance pattern (≥1 µg/mL), 60% had intermediate susceptibility (0.5 µg/mL) and 17% showed caspofungin susceptibility (≤0.25 µg/mL) (**Table 10**). The screening criteria given in the latest recommended guidelines (CLSI M27-S4) were only applied to samples showing resistance to caspofungin in susceptibility tests and also having higher growth rates than that for the SC5314 reference strain (Figure 10). Three clinical isolates were discarded from this group as they were susceptible to micafungin in experiments involving CLSI M27-A3 methodology and isolate UR78 was included which was resistant to micafungin in these assays (≥1 µg/mL) as well as having intermediate susceptibility to caspofungin (0.5 µg/mL).

Figure 10. Scheme explaining which isolates were included for CLSI M27-S4

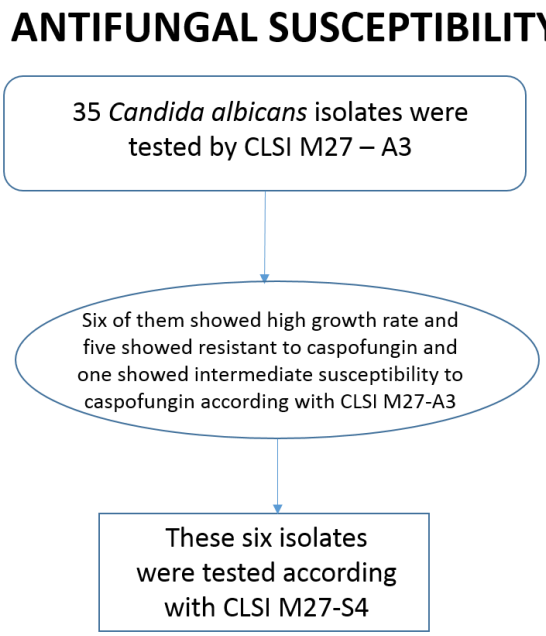


Table 10. Antifungal susceptibility to caspofungin according to CLSI M27-A3 and micafungin according to CLSI M27-S4

Strain	MIC CSF	MIC MCF
SC5314	≤ 0.25µg/mL (S)	≤ 0.25µg/mL (S)
UR475	≥ 1µg/mL (R)	≤ 0.25µg/mL (S)
UR78	0.5µg/mL (I)	≤ 0.25µg/mL (S)
UR527	2µg/mL (R)	≤ 0.25µg/mL (S)
UR242	≥ 1µg/mL (R)	≤ 0.25µg/mL (S)
UR147	≥ 1µg/mL (R)	≤ 0.25µg/mL (S)
UR608	**≥ 1µg/mL (R)**	**0.5µg/mL (R)**
UR600	≥ 1µg/mL (R)	
UR107	≥ 1µg/mL (R)	
UR390	≥ 1µg/mL (R)	
UR48	0.5µg/mL (I)	
UR393	0.5µg/mL (I)	

UR404	0.5µg/mL (I)
UR263	0.5µg/mL (I)
UR388	0.5µg/mL (I)
UR425	0.5µg/mL (I)
UR230	0.5µg/mL (I)
UR221	0.5µg/mL (I)
UR213	0.5µg/mL (I)
UR481	0.5µg/mL (I)
UR408	0.5µg/mL (I)
UR6	0.5µg/mL (I)
UR10	0.5µg/mL (I)
UR29	0.5µg/mL (I)
UR317	0.5µg/mL (I)
UR385	0.5µg/mL (I)
UR144	0.5µg/mL (I)
UR219	0.5µg/mL (I)
UR143	0.5µg/mL (I)
UR111	0.5µg/mL (I)
UR282	0.5µg/mL (I)
UR5	≤ 0.25µg/mL (S)
UR41	≤ 0.25µg/mL (S)
UR407	≤ 0.25µg/mL (S)
UR8	≤ 0.25µg/mL (S)
UR118	≤ 0.25µg/mL (S)

Note: MIC: minimal inhibitory concentration, CSF: caspofungin, MCF: micafungin, FLU: fluconazole, S: susceptible, I: intermediate, R: resistant

It was observed that isolate UR608 consistently had echinocandin resistance values (≥ 0.5 μg/mL) according to CLSI M27-S4 (**Table 10**). Susceptibility patterns for fluconazole from the previously selected group according to CLSI M27-A3 methodology showed that isolate UR475 was resistant to fluconazole (≥ 64 μg/mL) and isolate UR78 was susceptible dose dependent (SDD), i.e. susceptibility depended on maximum antifungal concentration in the bloodstream of a patient having normal renal function (≥ 32 μg/mL); however, these isolates did not have a fluconazole resistance pattern according to CLSI M27-S4 methodology (**Table 11**).

Table 11. Antifungal susceptibility to fluconazole according to CLSI M27-A3 and according to CLSI M27-S4

Strain	MIC FLU	MIC FLU*
SC5314	≤ 0.25μg/mL (S)	≤ 0.25μg/mL (S)
UR475	64μg/mL (R)	≤ 0.25μg/mL (S)
UR78	32μg/mL (R)	≤ 0.25μg/mL (S)
UR527	2μg/mL (S)	≤ 0.25μg/mL (S)
UR242	≤ 0.25μg/mL (S)	≤ 0.25μg/mL (S)
UR147	≤ 0.25μg/mL (S)	≤ 0.25μg/mL (S)
UR608	≥ 1μg/mL (S)	≤ 0.25μg/mL (S)

Note: MIC: minimal inhibitory concentration, CSF: caspofungin, MCF: micafungin, FLU: fluconazole, S: susceptible, I: intermediate, R: resistant, FLU* antifungal susceptibility according CLSI M27-S4.

2.2. Characterising the *FKS1* gene mutation pattern

The sequencing results were analysed using CLUSTALW software and compared to GenBank reference sequences (accession No. D88815). No mutations were found in the aforementioned regions after analysing both resistant strain sequences.

3.3. Discussing the susceptibility test

The susceptibility tests provided significant information regarding follow-up and control of nosocomial infections and constantly aided a patient's medical management [55]. One of the main challenges in clinical practice consists of obtaining reproducible results in laboratories which have completed the standardisation of these tests [98]. Developing and standardising these tests involves delineating clinical break-points defining susceptibility or resistance [80, 98].

Regarding the echinocandins, there has been a gradual change in defining the break-points; a *C. albicans* strain was initially considered resistant if a break-point was ≥ 2 μg/mL (Clinical and Laboratory Standards Institute methodological guidelines 2008) [96] whereas this break-point was reconsidered in 2011 as work by Pfaller *et al*., 2011 showed that the break-point until then in force detected *C. albicans* strains having 15% to 45% mutations in the *FKS1* gene. Using a break-point having ≥ 1 μg/mL resistance, 0.5μg/mL intermediate susceptibility and ≤0.25 μg/mL susceptibility led to increased 85%-95% detection of strains having mutations in the *FKS1* gene [80]. Towards the end of 2012, and backed by several pieces of research comparing the reproducibility of results in different laboratories, the Clinical and Laboratory Standards Institute's advisory committee on this topic evaluated consistency in reports when caspofungin was used in a standardised manner. When its results were analysed it was decided to define resistance as ≥ 0.5 μg/mL and susceptibility as ≤0.25 μg/mL for echinocandins using micafungin or anidulafungin as test antifungals [98, 99]. The foregoing indicates that the behaviour pattern and evaluation for proving echinocandin efficacy is very

dynamic, as the values related to the clinical break-point defining this fungi's susceptibility was re-evaluated three times in just 4 years.

In the present case, changes in interpretation values for applying different protocols to a group of clinical strains were effectively documented, showing (as previously reported) that applying CLSI M27-A3 guidelines led to reduced susceptibility values for caspofungin in practically 83% of the clinical samples evaluated; however, when using CLSI M27-S4 guidelines, just one clinical isolate was consistently resistant to echinocandins regarding both methodologies. A similar situation occurred regarding fluconazole susceptibility tests as CLSI M27-A3 guidelines led to two clinical isolates consistently having reduced susceptibility (**Table 11**), yet these isolates were susceptible when applying CLSI M27-S4 guidelines. Such findings from comparing both protocols merits a more detailed evaluation, i.e. regarding the role the solvent plays in these drugs' *in vitro* stability or their interactions in the test, as this is one of the key differences in applying protocols: water is used in CLSI M27-A3 and dimethyl sulfoxide (DMSO) in the CLSI M27-S4 guidelines.

Another key aspect worth highlighting regarding the results obtained refers to an isolate consistently being resistant to echinocandins in both methodologies that has not been previously directly exposed to this group of antifungals, i.e. the patient from whom this isolate was taken had not received any treatment related to echinocandins. This pattern led to inferring that (after reviewing the topic) multiple mechanisms operate simultaneously when resistance patterns are produced [14, 67]. Even though the aforementioned isolate had not been submitted to direct selective pressure from an echinocandin, other factors could have affected such type of pattern, such as the patient from whom the sample was taken had been

hospitalised in an ICU, implying a significant selective pressure [55]. Selective pressure in ICUs is associated with the prophylactic use of antifungals [126, 127], in some way initially explaining a nosocomial microorganism having this susceptibility profile. A further aspect worth considering is the clonal pattern regarding the spread of nosocomial infection implying phenotypic burden of hospital flora, as some work has described up to 58% of infection in an ICU caused by *Candida albicans* having this type of pattern [128] .

Another relevant finding regarding this isolate having resistance by both methodologies was the absence of point mutations in both regions of the *FKS1* gene traditionally described in the literature as the cause of resistance to echinocandins [15, 59, 74]. Such situation reinforces the concept of a response to cell stress caused by antifungals destabilising the cell wall according to their mechanism of action and revealed how chitin content becomes higher in *C. albicans* strains having reduced susceptibility patterns or resistance to echinocandins in the absence of *FKS1* gene mutation [129]; chitin synthesis is thus a mechanism for stabilising the cell wall resulting in such susceptibility profile [36, 119]. Phenotypical features must then be explored; one of the variables to consider refers precisely to chitin concentration compared to clinical reference strain SC5314.

Chapter IV - Nosocomial isolates' phenotypical traits

This chapter gives the results for the group of nosocomial isolates complying with the definition of nosocomial infection [50] bearing in mind that being nosocomial microorganisms their condition possibly differs from traditional descriptions [128] involving reference strains, laboratory strains or descriptions of clinical isolates from community-acquired *C. albicans* infections.

The results come from conventional assays describing the type of isolates mostly coming from sites involving large quantities/use of antifungal drugs [55, 130], as well as patients' critical conditions making Candida one of the relevant factors affecting patient mortality [5].

3.1. Growth rate

Growth rate was one of the first variables to be considered in phenotypical characterisation, from clinical reasoning related to mortality in late diagnosis and treatment of Candidiasis as, according to Morrel *et al*., (2008) the probability of dying 12 hours after starting antifungal therapy for candidaemia is 2.09 times more than that for a patient receiving it 12 hours before treatment for such identified infection (1.53-2.84 95%CI: p=0.018) [131]. After reviewing the literature referring to growth rate and association with resistance, work on resistance structure formation, such as biofilm, by Baillie and Douglas (1998) stated that resistance being produced in these cells at constant amphotericin B dose was independent of growth rate [132]. This only applied in the analysis of resistance for cells directly located in the biofilm structure.

Analysing bioscreen results involving planktonic cells, after processing all nosocomial samples, revealed that several isolates' growth curves during the 12 hour observation were faster than those for the SC5314 clinical reference strain. The clinical samples were grouped according to the following characteristics for comparison. The first group consisted of clinical isolates from patients who died as an outcome of their disease; parametric ANOVA was used for evaluating means repeated in reading growth for twelve hours, using Bonferroni test for adjusting multiple comparisons of means. The difference in means was considered significant at 0.5 regarding the SC5314 clinical reference strain; isolates complying with such characteristics were UR10, UR147, UR230, UR242 and UR393, accounting for 45% of the isolates associated with patients' death regarding clinical outcome. The second group consisted of isolates having reduced susceptibility to echinocandins in the susceptibility test using M27-A3 methodology; isolate UR 608 came from this group, showing resistance to echinocandins with both this methodology and M27-S4. The isolates having significant difference regarding growth curve (see **Figure 11**) were UR78, UR475, UR527, UR242, UR147 and UR608 accounting for 66% of the isolates having reduced susceptibility to caspofungin. Comparing CLSI M27-A3 methodology to CLSI M27-S4 methodology, the only clinical isolate consistently resistant to echinocandins (UR608) had a significant difference in means regarding the growth curve.

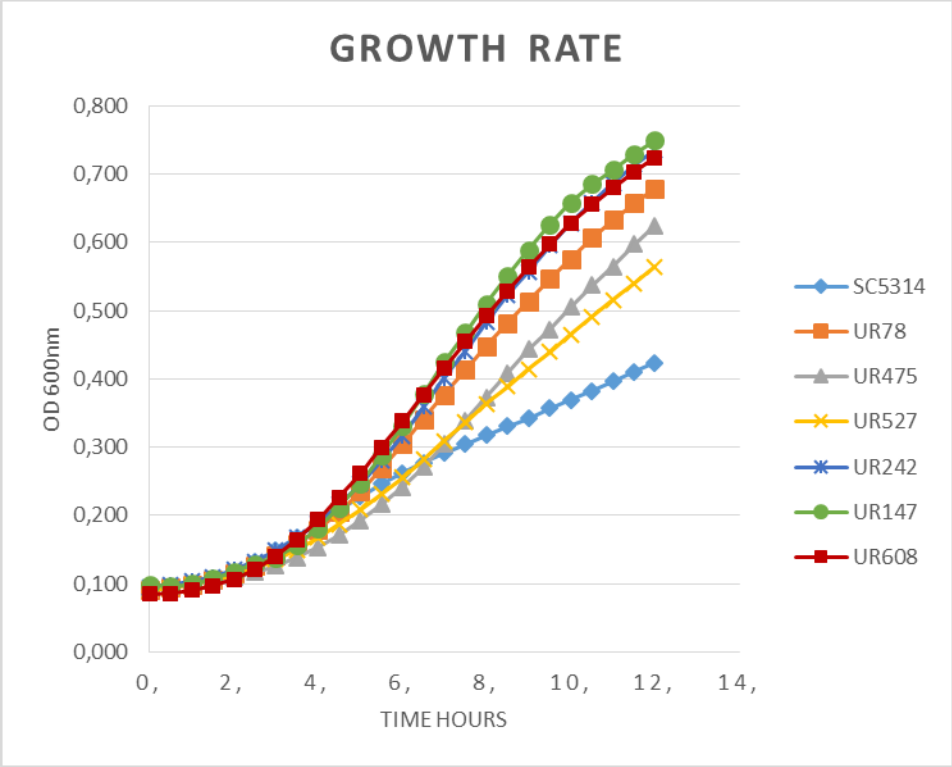


Figure 11. Growth curves for nosocomial isolates compared to that for reference strain SC5314.

Growth curves were determined with a Bioscreen apparatus (Life Sciences). Planktonic cell growth was determined at 30°C in RPMI media for 12 hours [115].

It is worth mentioning UR147 isolate having faster curves regarding reference strain SC5314 growth as it had relation with reduced susceptibility regarding M27-A3 methodology and was also associated with the group where the patient from whom the sample was taken died. The other isolates worth mentioning were UR242 which had the same pattern with UR147 and UR 608 having reduced susceptibility in both methodologies and significant differences regarding growth curves compared to the SC5314 clinical reference strain.

The growth rate is a differential factor in these nosocomial isolates, even though a similar description could not be found in the pertinent literature, cell wall instability leading to activating the MAPK pathways of fungi has been described [133]. The situation described

for such signalling pathways could affect these isolates' metabolism, as increased growth rate on being submitted to metabolism-related stress conditions has been reported in other yeast models (*Saccharomyces cerevisiae*) [134]. This work highlighted two genes associated with cell wall activity (*NRG1*, *GSL2*); these genes are reported in this article in the supplementary material as genes associated with increased growth rate [134]. The results from this assay led to inferring that these isolates on being exposed to antifungals in a hospital setting [127] underwent selective pressure frequently affecting the stability of structures like cell membrane and wall. The foregoing can be supported by Sorgo *et al*.'s findings (2011) stating that the azoles frequently used in medical practice affect cell wall stability [16]. The results described here underline the need for further studies focused on better elucidating this phenotypical feature.

3.2. Cell adhesion and biofilm formation

Cell adhesion and biofilm formation are important phenotypical features which are related to *C. albicans* virulence; its clinical importance lies in the ease of becoming established in medical devices frequently used in patient care, thus becoming a focus of infection for a patient [71]. Biofilm formation is also associated with one of the mechanisms generating resistance to antifungals and thus becomes one of the factors associated with the distance that pathogenous microorganisms compromising patients' health can spread [71]. The following results refer to cell adhesion and biofilm formation in the group of selected clinical isolates.

3.2.1. Cell adhesion

Figure 12 gives cell adhesion assay results highlighting the reduced values for isolates UR147, UR78, UR600 and UR475. These isolates came from the group having reduced susceptibility to caspofungin according to M27-A3 methodology and an interesting interpretation when considering that biofilm promotes the development of antifungal tolerance mechanisms [73]. Another result concerned isolates UR78 and UR475 consistently giving extremely reduced cell adhesion values which, according to M27-A3 methodology, showed tolerance to fluconazole, i.e. having reduced susceptibility to two groups of antifungals. A review of the pertinent literature by Sorgo *et al.*, (2011) refers to how azole exposure alters cell wall stability and one change referred to a decrease of proteins such as Als3 and Hwp1, which are important in cell adhesion [16, 135, 136].

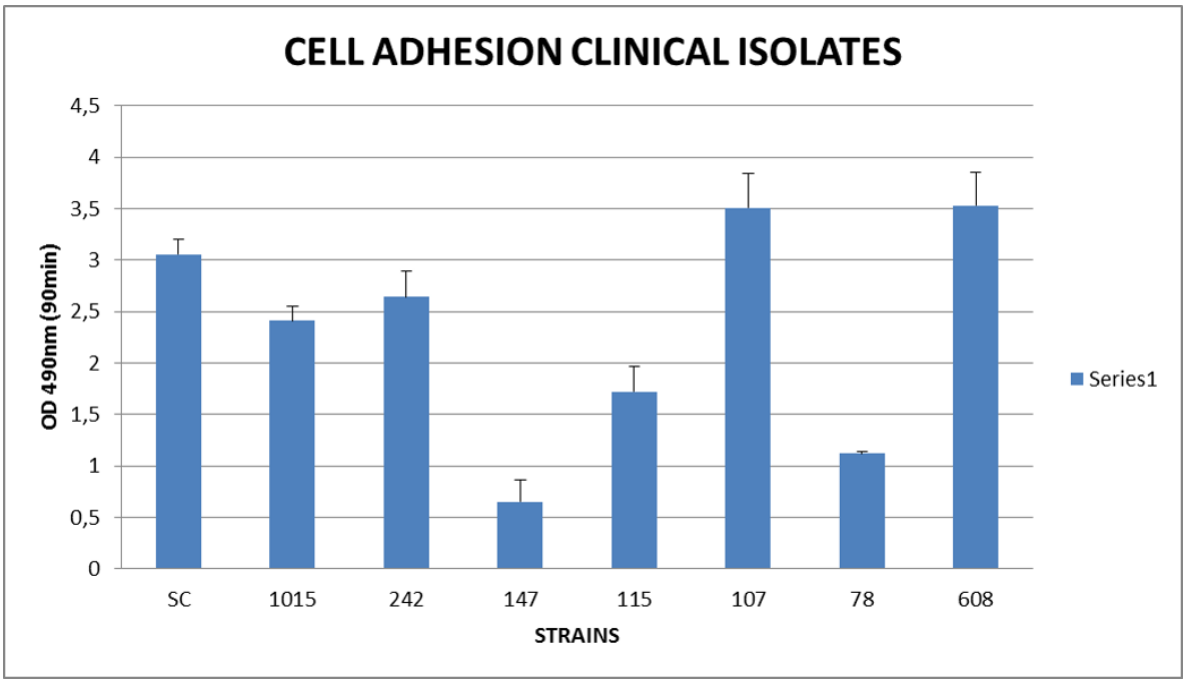


Figure 12. Cell adhesion in clinical isolates with reduced susceptibility**.**

Several clinical isolates displayed low cell adhesion when compared to the reference strain SC5314. Reference strain and clinical isolates were incubated at 37ºC in RPMI 1640 MOPS on 96-well polystyrene plates for 90 min. Data are shown as XTT measurements at 490nm. Standard deviations were calculated from three independent measurements [114].

3.2.2. Biofilm

Figure 13 gives the biofilm formation test results, again highlighting isolates UR147, UR78 and UR475 thereby agreeing with the resistant reference strain, identified as R1015, where cell concentration at the end of the assay was lower compared to the SC5314 clinical reference strain. A change in the pattern for the R1015 resistant reference strain was observed in this assay, compared to cell adhesion test results where there was a difference at lower concentration regarding the amount of cells obtained when taking the reading.

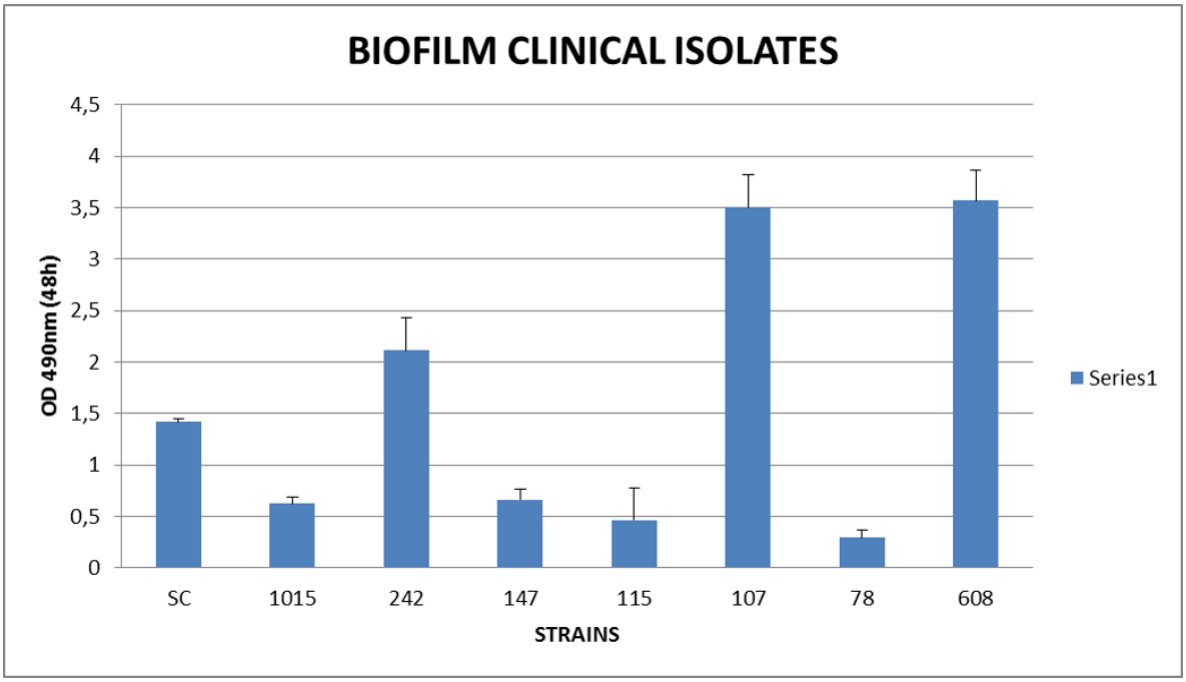


Figure 13. Biofilm formation in clinical isolates having reduced susceptibility.

Several clinical isolates displayed low biofilm formation when compared with the reference strain SC5314. Reference strain and clinical isolates were incubated at 37ºC in RPMI 1640 MOPS on 96-well polystyrene plates for 48 hours. Data are shown as XTT measurements at 490nm. Standard deviations were calculated from three independent measurements [114].

Another of the changes observed referred to clinical isolate UR608 having an echinocandin resistance pattern in M27-A3 and M27-S4 tests, giving greater concentration of cells forming biofilm compared to the SC5314 clinical reference strain. When comparing isolate UR608 with results from literature, similar findings by Angiolella *et al*., (2008) reported increased

biofilm formation in a resistant laboratory strain [85]. However, these results contrasted with those concerning resistant reference strain R1015 which had lower biofilm-forming cell concentration in these assays.

These assay results defined two groups of isolates compared to the SC5314 clinical reference strain. Isolates which had shown reduced susceptibility to echinocandins were placed in the first group, along with isolates UR475 and UR78 having reduced susceptibility to fluconazole. Correlation with the pertinent literature also refers to changes in these isolates' cell wall where the activity of proteins such as Als3 and Hwp1 was affected, thereby explaining less biofilm formation [16]. By contrast, the UR608 clinical isolate had a greater concentration of biofilm forming cells, the explanation referring to remodelling cell wall structural components having a greater amount of glucoproteins, thereby promoting the formation of this structure [85].

The results concerning the pattern of both groups of clinical isolates were related to an evolutionary ability to adapt to the nosocomial conditions in which they had grown and respond to a selective pressure effect where the pattern regarding factors associated with biofilm formation could be opposed, as the biofilm formation pattern for one group might be low but be high for another isolate. Pertinent literature supports such results for both situations [55, 137].

3.3. Colony morphology

Figure 14 shows representative pictures in the colony morphology test on plates in hyphal-inducing media conditions, i.e. foetal serum, SPIDER medium and SLD medium supplemented with methionine [115, 138]. The purpose of these culture media was to evaluate *C. albicans* morphological transition from yeast to hypha; the information obtained according to the colonies' morphology led to inferring the predominant morphology, as well as these cells' invasion capacity. Two extremes were identified regarding the morphology of the colonies' structure: "wrinkled" and "smooth" colonies; wrinkled colonies had ridged colonies indicating the presence of cells in the form of yeast, pseudohyphae and hyphae.

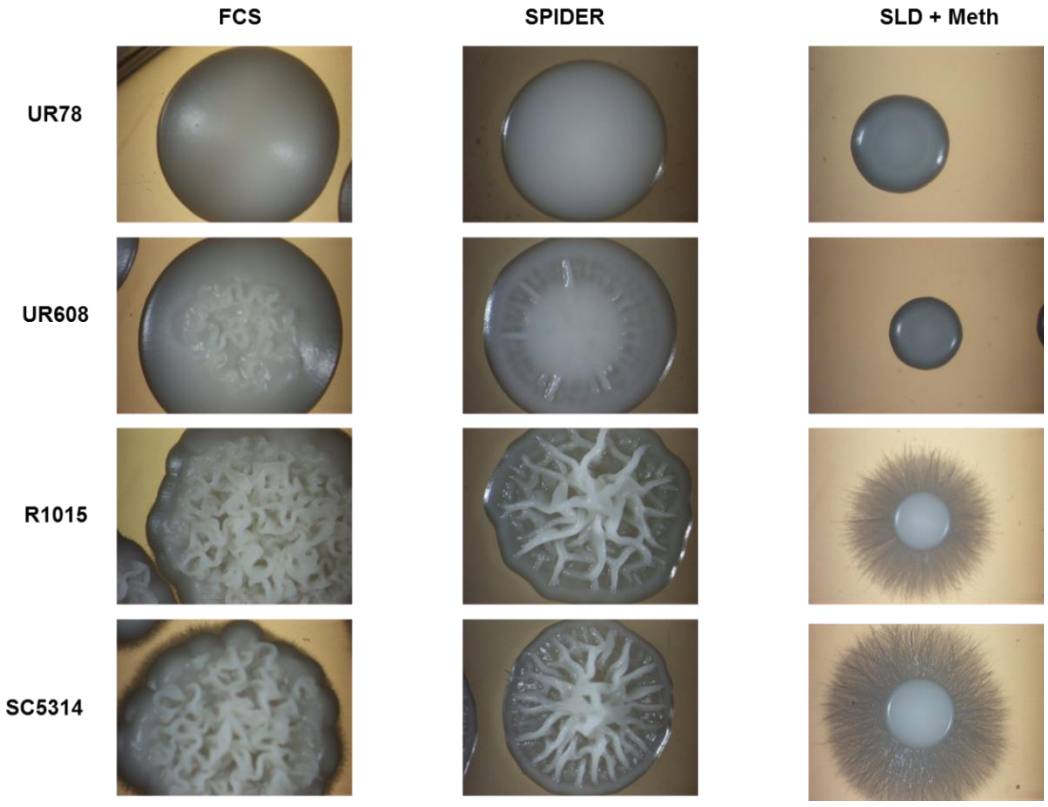


Figure 14. Colony morphology in inducing media.

Foetal calf serum (FCS), Spider and SLD + Methionine supplemented media. UR78 and UR608 clinical isolates were compared with reference strains R1015 and SC5314.

Their texture resembled that of rubber due to the amount of extracellular matrix deposition. Smooth colonies had a dome form essentially consisting of yeasts having a pasty consistency without extracellular matrix (**Figure 14**) [139].

The following findings arose from comparing the SC5314 clinical reference strain to our sample of clinical isolates read after 7 days incubation at 30ºC; 70% of the isolates had smooth dome morphology in FCS, 27% of them had minimum peripheral invasion and incipient formation of wrinkled colonies in one isolate (UR608). Regarding SPIDER induction medium where mannitol provided the primary carbon source, 33% of the isolates had smooth dome morphology, 60% had minimum peripheral invasion and 7% initial formation of wrinkled colonies. SLD medium supplemented with methionine led to 83% of the isolates having colonies whose morphology was smooth dome and the remaining 17% minimum peripheral invasion.

It was observed that isolate UR78 did not show filamentation in the media used; this pattern was the same shown by isolate UR475 (included in the photograph in **Figure 28** below); these two isolates showed reduced susceptibility to fluconazole and caspofungin in CLSI M27-A3 tests. Crossing this information with biofilm formation assay results, these two isolates had the lowest biofilm-forming cell concentrations in 48 hour reading. The opposite was found for isolate UR608, correlating with corresponding susceptibility patterns explored with the CLSI M27-A3 guidelines showing resistance to caspofungin and M27-S4 guidelines showing resistance to micafungin, i.e. this isolate consistently showed resistance to echinocandins in both methodologies and was the only one showing a slight degree of filamentation in foetal serum medium and SPIDER medium. However, no filamentation was

observed in SLD medium supplemented with methionine (**Figure 14**). Crossing this information with biofilm formation test results it was observed that isolate UR608 had greater biofilm-forming cell concentration when read at 48 hours compared to the SC5314 clinical reference strain (**Figure 13**). The amount of cells obtained when read at 90 minutes for cell adhesion assays revealed no significant differences for this isolate (**Figure 12**).

Regarding filamentation results and biofilm-forming cell concentration reading, a reference strain resistant to echinocandins having a mutation identified in HS1 (R1015) was also used as reference pattern. This resistant reference strain (R1015) had filaments in all culture media used; however, it had low cell concentration in the biofilm assay read at 48 hours when compared to the SC5314 clinical reference strain (**Figure 13**). Comparing this phenotypical feature in reference strain R1015 to the clinical isolates, they shared filamentation with isolate UR608 regarding the formation of these structures in culture in foetal serum medium and SPIDER medium, even though the amount of filaments observed in these media was lower for the clinical isolate. Concerning SLD medium supplemented with methionine, clinical isolate UR608 did not make filaments (**Figure 14**). Comparing biofilm-forming cell concentration read at 48 hours, the R1015 echinocandin resistant reference strain had a similar pattern to that observed for isolates UR78 and UR475, in turn, being lower than that for the SC5314 reference strain (**Figure 13**). Comparing filament formation for the R1015 resistant reference strain and the SC5314 clinical reference strain observed in SLD medium supplemented with methionine, R1015 resistant reference strain had shorter and less filaments (**Figure 14**), possibly explaining this reference strain's lower biofilm-forming cell concentration.

The information from the previous results is important as several authors consider that one of the main attributes of *C. albicans* virulence is related to its ability regarding dimorphic transition from yeast to hyphae and vice versa [34, 85]. Hyphae constitute a major structural part in early biofilm and maturation stages, accompanied by resistance, pathogenicity and infection persistence described in the relevant literature [73, 114]. According to filamentation assay results, the absence of filaments in SLD medium supplemented with methionine for the isolates of interest (UR78, UR475 and UR608) showed Gpr1 receptor inability to activate the cAMP-PKA pathway where one of the main effects lies in inducing filament-associated genes, i.e. *HWP1* (**Figure 15**) [138, 140].

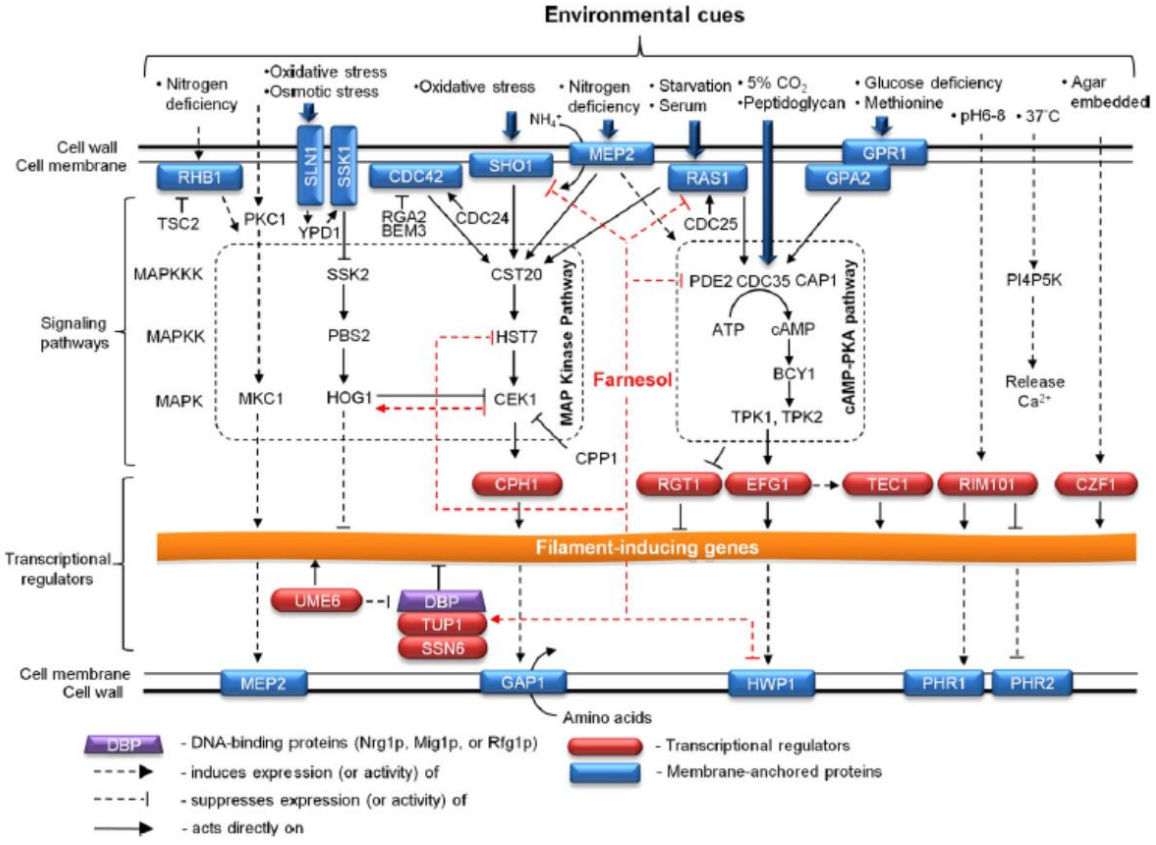


Figure 15. Signalling pathways and environmental factors affecting *C. albicans* filamentation (Han *et al*., 2011).

Two different signalling pathways affect filamentation: the MAP Kinase pathway and the cAMP – PKA pathway.

The foregoing results could lead to stating that the signalling pathway mediated by this receptor (Grp1) was compromised regarding these clinical isolates; however, wrinkled cell formation in FCS and SPIDER media was observed for isolate UR608, predominantly having to do with the other signalling pathway -MAPK– whose filament inducing gene is *GAP1* [140-142]. This pattern could lead to inferring that this clinical isolate has a compensation mechanism via this pathway.

Filamentation is a response to a series of predominantly external factors seeking to guarantee cell viability in the variable conditions of the setting in which they find themselves or which are the manifestation of adaptation to a variable setting [143, 144]. DNA binding proteins (Nrg1, Mig1 and Rfg1) inhibiting filamentation have been described from two signalling pathways (MAPK and cAMP-PKA) whose target genes are *ECE1* and *HWP1* [140, 145]. Tup1 and Ssn6 represent other repression factors regarding filamentation [140]. Homann *et al*., (2010) described a regulatory network governing morphogenesis and invasion in this microorganism, referring to post-transcriptional control with negative regulators such as Nrg1 and Tup1 as well as positive regulators Tec1 and Gat2 [139].

Filament formation and biofilm formation thus involve a complex system of control affected by master transcriptional biofilm regulators described in elegant work that include Bcr1, Tec1, Efg1, Ndt80, Rob1 and Nrg1 involved in a different level of expression in a biofilm regulatory network, in addition to the already mentioned effector signalling pathways [139, 146]. The forgoing constitutes a complex system revealing differing responses in clinical isolates, highlighting the need for further studies aimed at explaining the pattern observed for

isolates having crossed resistance in CLSI M27-A3 methodology and isolates consistently being resistant to echinocandins by M27-S4 methodology.

3.4. Gene expression

The expression of genes for this group of clinical isolates was focused on genes related to cell wall stability which, according to the pertinent literature, is described within mechanisms of response to cell stress caused by caspofungin [36, 84, 86, 87, 133]. Genes were selected for this initial assay, considering functional aspects such as filamentation (*ERG5*, *FKS1* and *PNG2*); a second group of genes was related to response to exposure to echinocandins (*SKO*, *MKC1* and *CNB1*) whilst a third group of genes were GPI and related to cell wall stability, according to the description in the pertinent literature (*SOD5*, *PGA62*, *PGA13*, *PGA31* and *PGA23*). This was the first assay leading to establishing a hypothesis for explaining reduced echinocandin susceptibility in a group of nosocomial clinical isolates. More experimental work is needed for verifying such initial results. The *ERG5* gene was selected as it has been described in cases of cross-resistance between azoles and amphotericin B. Assays described regarding *S. cerevisiae*, a distant cousin of *C. albicans*, have shown that the deletion of this gene increases susceptibility to caspofungin [147, 148]. The isolates selected for this assay were those in the CLSI M27-A3 methodology which had reduced susceptibility to fluconazole and caspofungin regarding isolate UR475 and reduced susceptibility to fluconazole and micafungin for isolate UR78; isolate UR147 was selected as it had reduced susceptibility to caspofungin and was associated with the outcome of the death of a patient from whom such sample had been taken (**Figure 16**).

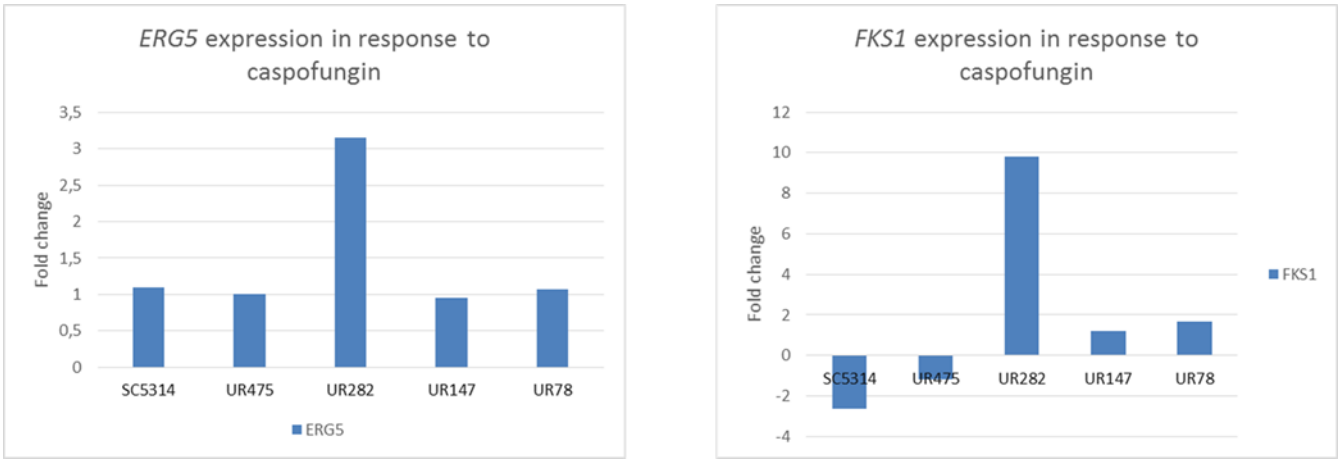


Figure 16. *ERG5* and *FKS1* expression in response to caspofungin.

Reference strain SC5314 and clinical isolates were incubated in RPMI 1640 MOPS at 37ºC for 24 hours in absence and presence of a sublethal MIC concentration of caspofungin (0.125µg/mL). Fold change expression between absence and response to caspofungin was calculated using ΔΔCt method. Each gene was normalised to *ACT1*.

The results of a first assay regarding relative RT-qPCR quantification of the selected genes revealed differences in clinical isolate and clinical reference strain SC5314 expression when submitted to a caspofungin concentration below MIC50 for 24 hours. Regarding the *FKS1* gene, it was observed that it was down-regulated in the reference strain; slight down-regulation, resembling the clinical reference strain pattern, was observed for isolate UR475 whereas the situation was totally different for isolates UR147 and UR78 as this gene's expression was not down-regulated (**Figure 16**). The pattern observed in the first case involving reference strain SC5314 and clinical isolate UR475 could be explained by caspofungin's mechanism of action altering cell wall integrity, thereby indicating compensatory response mechanisms against cell wall stress caused by the antifungal action on the cell wall [38]. Regarding isolates UR78 and UR147, there were no significant changes in gene expression for *ERG5*, in spite of cell wall stress caused by the presence of the

antifungal. The difference in expression observed in this test for the SC5314 and UR475 strains indicated that the antifungal agent might have a cell wall mechanism of action.

Reviewing the pertinent literature revealed that *CNB1* encodes the calcineurin regulatory subunit required for activating this signalling pathway, playing a significant role in cell wall stability and thereby acting on the *FKS1* gene via the *CRZ1* transcription factor [59]. Singh *et al.*, (2009) showed that this pathway's integrity promotes micafungin tolerance [72]; the pattern observed here led to inferring that this clinical isolate had different compensation mechanisms to those indicated for this signalling pathway as this isolate had reduced susceptibility to caspofungin regarding CLSI M27-A3 methodology. No significant differences were observed in this gene's expression regarding isolates UR78 and UR147 compared to the SC5314 clinical reference strain. Differential expression was observed in isolate UR475 with significant down-regulation in this gene's expression in exposure to caspofungin when compared to clinical reference strain SC5314 (**Figure 17**).

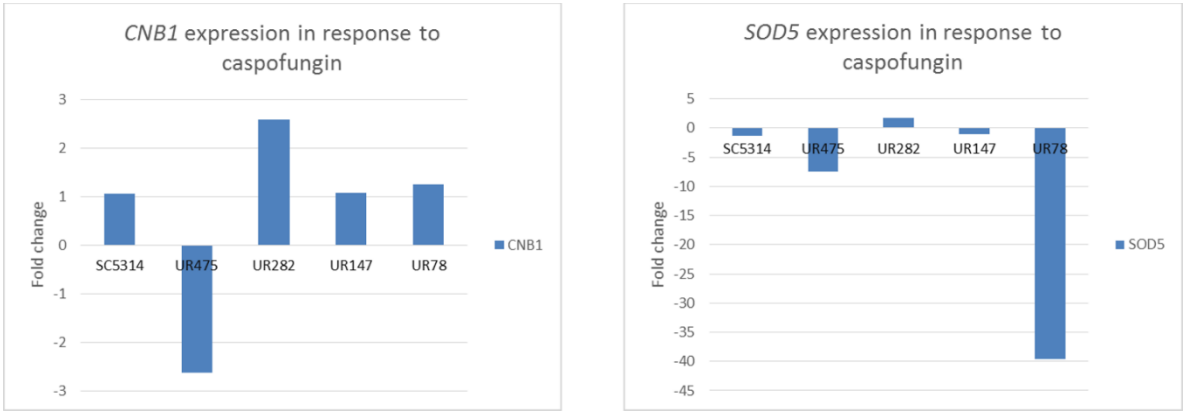


Figure 17. *CNB1* and *SOD5* expression in response to caspofungin.

Reference strain SC5314 and clinical isolates were incubated in RPMI 1640 MOPS at 37ºC for 24 hours in absence and presence of a sublethal MIC concentration of caspofungin (0.125µg/mL). Fold

change expression between absence and response to caspofungin was calculated using ΔΔCt method. Each gene was normalised to *ACT1*.

Concerning the *SOD5* (encoding a GPI -anchored cell wall protein), a difference was observed concerning this gene's expression becoming downregulated in isolates UR475 and UR78 compared to its expression in clinical reference strain SC5314 (**Figure 17**). Such down-regulation was much greater in isolate UR78. These two isolates shared a significant background showing reduced susceptibility to fluconazole and caspofungin. The pattern observed yet again indicated a cell stress mechanism for the *SOD5* gene, contrasting with work by Liu *et al.,* (2005) who demonstrated greater expression on being exposed to sub-lethal concentrations of caspofungin; however, their exposure time for this assay was 180 minutes whilst ours lasted 24 hours [38]. The foregoing indicated the need for further studies aimed at clarifying whether such strong down-regulation response is implicated in another compensation pathway regarding cell wall stability where exposure time plays a significant role in compensation response.

Another gene which showed changes in expression following the exposure to caspofungin was the *PGA23* gene encoding a cell wall GPI; this gene's expression was upregulated in *Candida albicans* clinical isolates UR475, UR78 and UR147, clearly showing differential expression compared to clinical reference strain SC5314 (**Figure 18**). A review of the pertinent literature showed that this gene's expression is involved in activating other genes maintaining cell wall integrity, such as *PGA13*. This expression profile is also related to transcription factors Cas5, Rlm1 and Ada2, all involved in maintaining cell wall stability against caspofungin stimulus [37]. Differential expression was also found for the *PGA13* gene where this gene was downregulated in clinical reference strain SC5314 and clinical

isolate UR475, contrary to clinical isolates UR147 and UR78 (**Figure 18**). The *PGA13* gene was also found to be related to cell wall integrity regarding exposure to caspofungin and its expression profile was more related to the Rlm1 transcription factor [37]. Downregulation in this case could indicate that transcription factors other than Rlm1 were more active for clinical reference strain SC5314 and clinical isolate UR475.

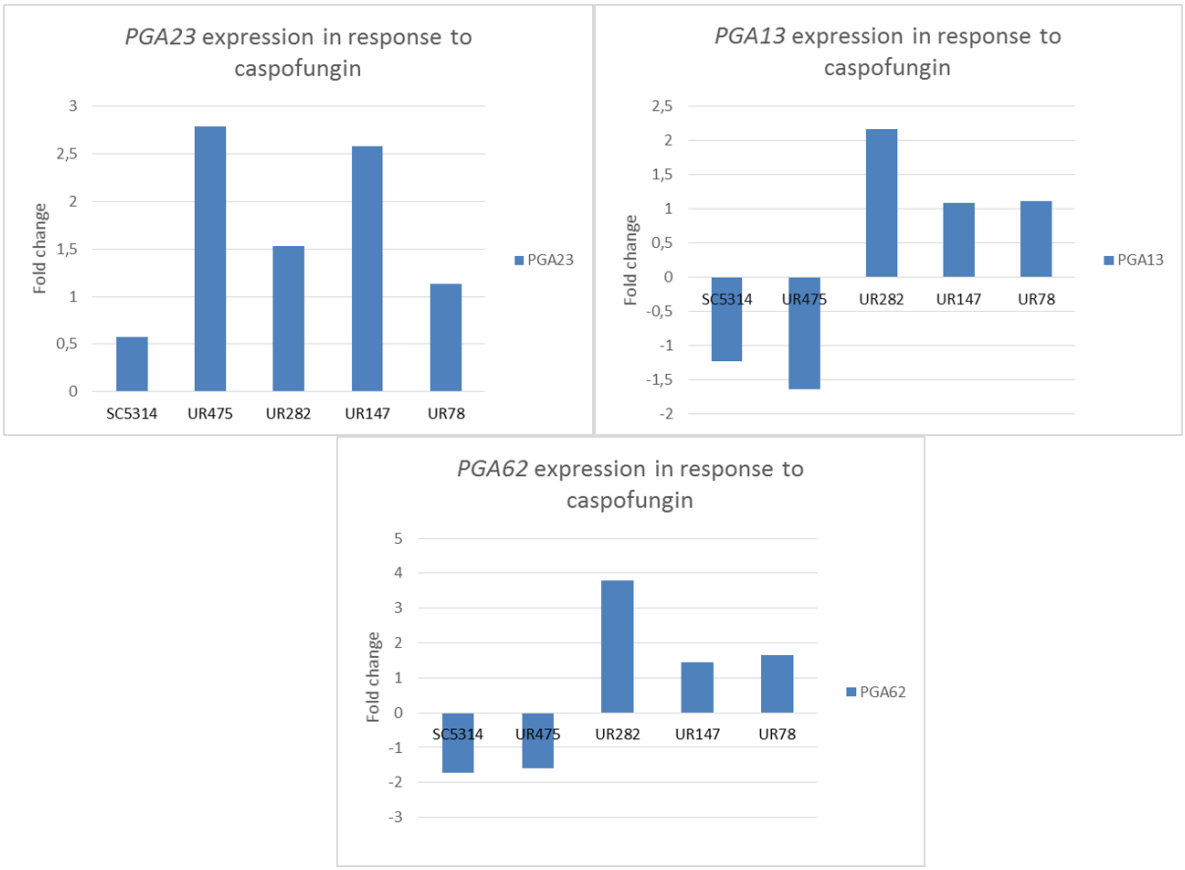


Figure 18. *PGA23, PGA13* and *PGA62* expression in response to caspofungin.

Reference strain SC5314 and clinical isolates were incubated in RPMI 1640 MOPS at 37ºC for 24 hours in absence and presence of sublethal MIC concentration of caspofungin (0.125µg/mL). Fold change expression between absence and response to caspofungin was calculated using ΔΔCt method. Each gene was normalised to *ACT1*.

Expression results for the *PGA62* gene revealed differences as this gene was downregulated in clinical reference strain SC5314 and clinical isolate UR475, different to expression in clinical isolates UR147 and UR78 (**Figure 18**). The mutant strain having double deletion of

the *PGA62* gene was resistant to caspofungin and also had increased chitin concentration in the cell wall as a compensation mechanism [36]. This finding indicated that the *PGA62* gene became downregulated in reference strain SC5314 and isolate UR475 as a compensation response to exposure to caspofungin.

Regarding the *PGA31* gene, it was found that it was downregulated in clinical reference strain SC5314 and *C. albicans* clinical isolates UR475 and UR147, unlike in *C. albicans* clinical isolate UR78 (**Figure 19**). Plaine *et al.*, (2008) showed that strains having double deletion of this gene had greater susceptibility to caspofungin and significantly reduced cell wall chitin content [36]. Regarding this finding and that reported in this assay, this would not have been the predominant compensation mechanism in the reference strain or in clinical isolate UR475.

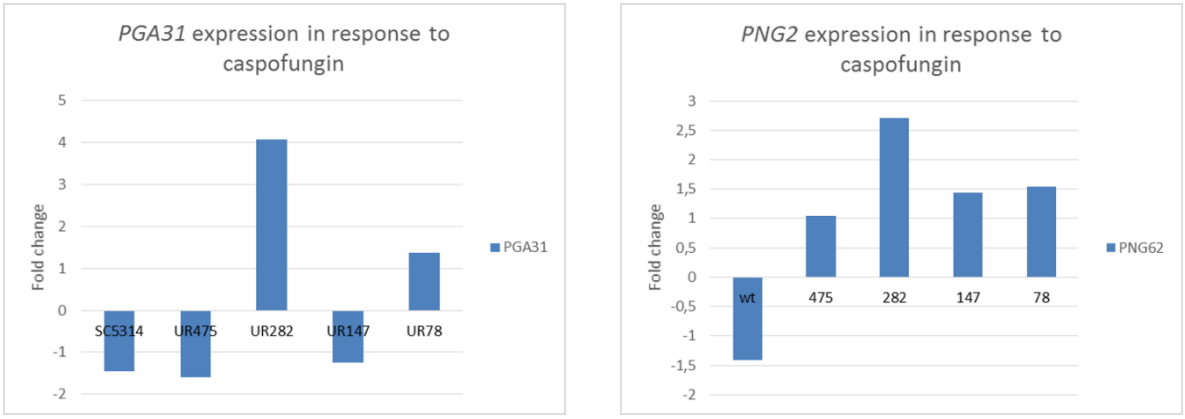


Figure 19. *PGA31* and *PNG2* expression in response to caspofungin.

Reference strain SC5314 and clinical isolates were incubated in RPMI 1640 MOPS at 37ºC for 24 hours in the absence and presence of sublethal MIC concentration of caspofungin (0.125µg/mL). Fold change expression between absence and response to caspofungin was calculated using ΔΔCt method. Each gene was normalised to *ACT1*.

PNG2 gene expression resulting from cell exposure to caspofungin was only downregulated in clinical reference strain SC5314 (**Figure 19**), unlike in clinical isolates UR475, UR147 and UR78; such difference was important as these clinical isolates' phenotypical pattern also differed from that of the reference strain as well as having a pattern of reduced susceptibility. According to the pertinent literature, this gene's expression becomes upregulated in modified strains which are resistant to fluconazole and amphotericin B [149] and have increased expression when *C. albicans* is exposed to sublethal concentrations of caspofungin [37]. Png2 is a protein which is located on cell surface and it has been estimated that one of its functions is implicated in cell adhesion; it has also been described as having a high rate of mutation to allow *C. albicans* adaptation to a host's setting [150]. Regarding the clinical isolates tested in the assay, further studies are required for a better understanding of the predominant signalling pattern in these samples as their nosocomial origin could be affected by factors influencing this protein's expression.

Completing exploration of the regulation pathways enabling cell wall stability to be maintained, differential expression was found for the *MKC1* gene, one of the key elements in the MAPK pathway (**Figure 14**). This gene was downregulated in clinical isolates UR475 and UR78, unlike in clinical reference strain SC5314 and clinical isolate UR147 (**Figure 20**). This gene's function within the framework of cell wall regulation has been explored in the pertinent literature, suggesting that it plays a compensatory role against cell wall stress induced by caspofungin. Munro *et al*., (2007) showed how deleting this gene induced a reduction in chitin content, this being the structural element maintaining cell wall stability [86, 151]. Such results should be considered regarding exposure time to caspofungin, as Markovich *et al*., (2004) used short exposure (10 minutes) unlike the assay reported here

which lasted 24 hours for the clinical isolates in question. This situation merits fresh studies using these clinical isolates to better define the sequence of actions integrated in the response to cell stress caused by caspofungin. Another aspect worth highlighting in this first work refers to the pattern of clinical isolates UR475 and UR78 coinciding, bearing in mind that they had both shown reduced susceptibility to caspofungin and fluconazole, according to M27-A3 methodology.

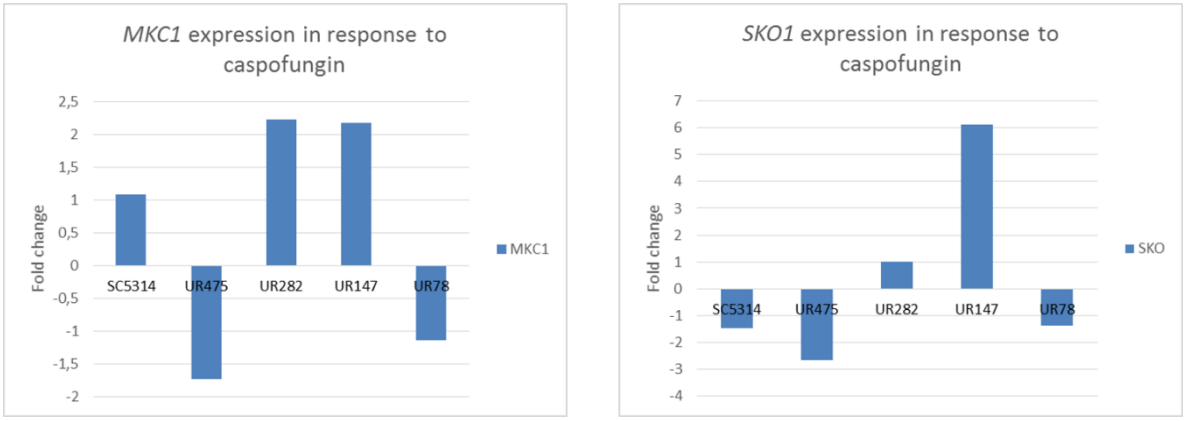


Figure 20. *MKC1* and *SKO1* in response to caspofungin.

Reference strain SC5314 and clinical isolates were incubated in RPMI 1640 MOPS at 37ºC for 24 hours in the absence and presence of sublethal MIC concentration of caspofungin (0.125µg/mL). Fold change expression between absence and response to caspofungin was calculated using ΔΔCt method. Each gene was normalised to *ACT1*.

Concerning exploration of gene expression against cell stress imposed by caspofungin, it was found that the *SKO1* gene was downregulated in clinical reference strain SC5314 and clinical isolates UR475 and UR78, unlike in clinical isolate UR147 (**Figure 20**). Within the framework of signalling and response to cell wall stress, the *SKO1* gene forms part of the high osmolarity glycerol response (HOG) signalling pathway implicated in maintaining cell stability through high chitin concentration as a structural element stabilising the *C. albicans* wall [87, 151].

The variation observed in the clinical isolates and the reference strain showed that exposure to caspofungin had an effect on the cell wall and that further studies are needed to provide a clearer picture of the compensation mechanisms intervening in these clinical isolates' phenotypical pattern.

3.5. Determining chitin content after caspofungin was added.

The sample selected in this assay consisted of clinical isolates which had reduced susceptibility to caspofungin, according to the CLSI M27-A3 guidelines. **Figure 21** shows the micrographs with fluorescence for clinical reference strain SC5314 and clinical isolates UR78 and UR608. The clinical isolates were exposed for 6 hours to a 0.025 µg/mL caspofungin concentration; the results analysed using ImageJ software revealed significant differences in mean reading of intensity from simple random sampling of all the cells observed in the microscope field.

A selected sample size of 1,787 cells, taken from a total population of 2,935 cells involving the clinical reference strain and the clinical isolates, was used for comparing each subgroup which had not been exposed to caspofungin to the corresponding subgroup which had been exposed to caspofungin, as well as comparing them to clinical reference strain SC5314. The statistical analysis consisted of univariate analysis; homogeneity of variance by Levene test was 0.000 and Welch and Brown-Forsythe tests for measuring equality of means gave 0.000, thereby showing significance.

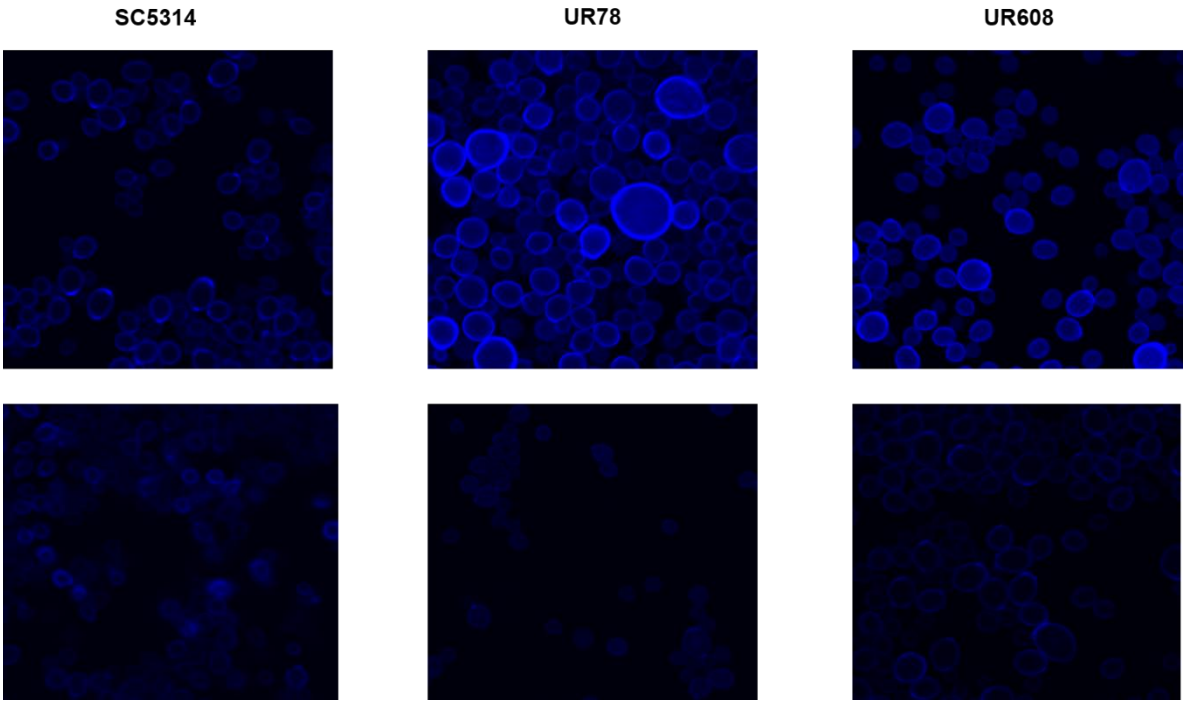


Figure 21. Chitin content.

Fluorescence intensity for the reference clinical strain SC5314 versus clinical isolates tested with Caspofungin at 0.25 µg/mL in the upper row; controls without caspofungin are shown in the lower row.

Comparing reference strain mean reading intensity to that for the clinical isolates which had not been stimulated by caspofungin, only clinical isolate UR608 had a lower mean reading than the SC5314 clinical reference strain (**Figure 22**). The other isolates in the assay had higher mean readings than the reference strain; the foregoing agreed with interpretation of previous work indicating greater chitin concentration in the cell wall of these *C. albicans* clinical isolates compared to reference strain SC5314 [119, 152, 153]. Mean fluorescence reading increased when the reference strain and the clinical isolates were submitted to caspofungin pressure, indicating greater chitin concentration in the cell wall. Clinical isolate UR608 had a higher reading when submitted to caspofungin pressure compared to reference strain SC5314; the same pattern was observed in the other clinical isolates where mean

readings were higher when compared to a the clinical reference strain and the differences were minimal in just two cases (UR242 and UR527) (**Figure 22**). **Figure 23** shows clinical isolates UR78, UR147 and UR 475 whose expression patterns have been dealt with in the previous chapter; their results are given in terms of mean readings of fluorescence at higher chitin concentration as compensation mechanism regarding cell wall stress produced by exposure to caspofungin compared to that for clinical reference strain SC5314.

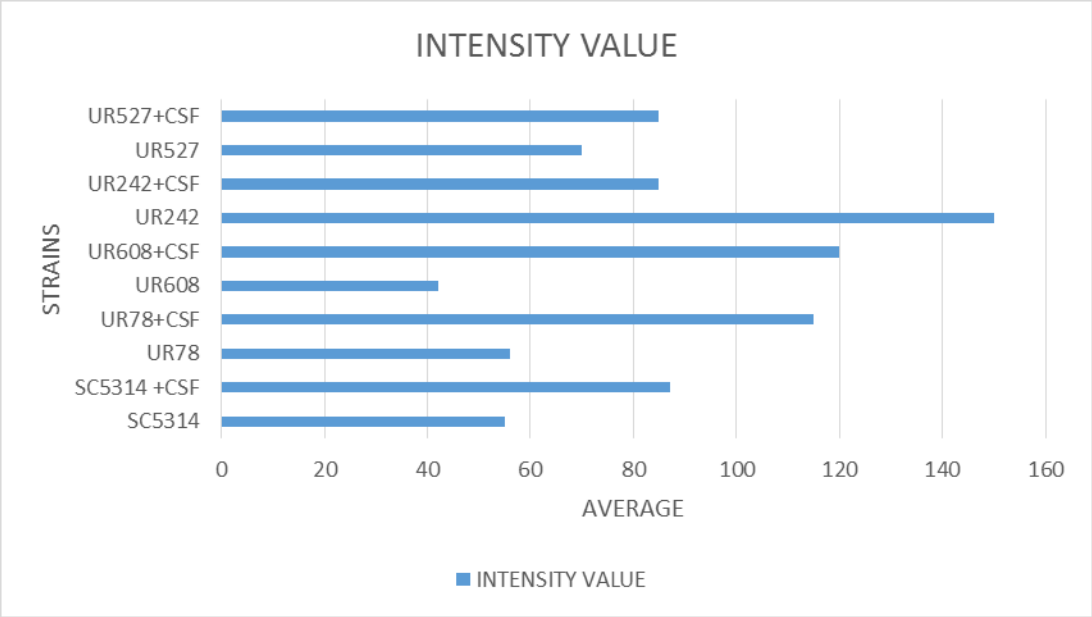


Figure 22. Graphic fluorescence intensity.

Average values were compared between reference clinical strain SC5314 and clinical isolates in absence or presence of caspofungin (CSF).

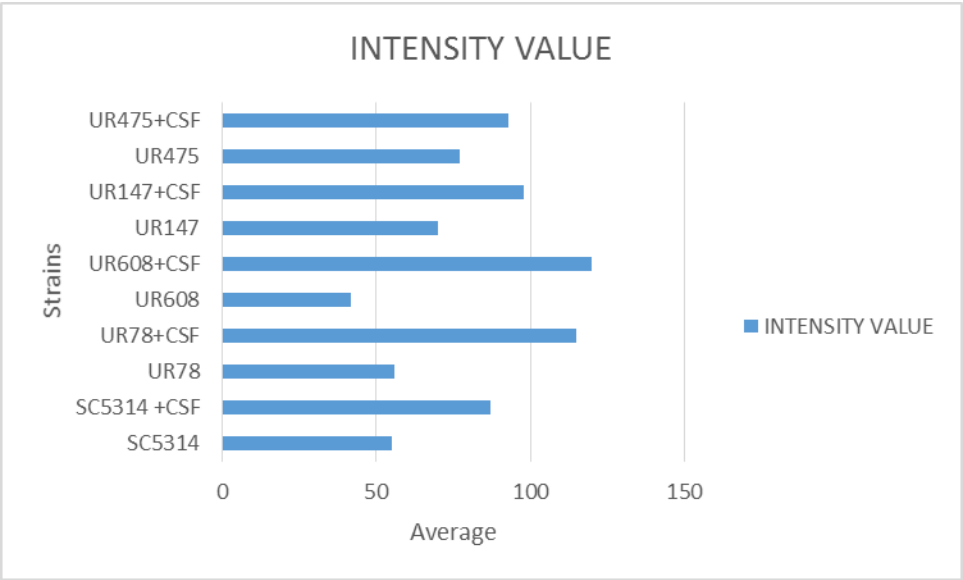


Figure 23. Graphic of fluorescence intensity.

Average values were compared between reference clinical strain SC5314 and clinical isolates in absence or presence of caspofungin (CSF).

These results for the clinical isolates showed that one of the mechanisms explaining reduced susceptibility to caspofungin according to CLSI M27-A3 guidelines was concerned with chitin concentration in the cell wall [36, 119]. Concerning clinical isolate UR608, which was resistant according to both CLSI M27-A3 and CLSI M27-S4 guidelines, this would explain its pattern better, as no mutations could be found for the *FKS1* gene in the two hot spots which, according to the pertinent literature, provide the most frequently found explanation for this susceptibility pattern [59, 129]. These findings highlight compensation mechanisms and the significant role played by the signalling pathways involved in cell wall stability, such as the HOG, calcineurin, cAMP and MAPK pathways indicating that the main activation trigger for these pathways is cell wall stability, where the Fks1 and its catalytic subunit Rho also play a key role as most of the mechanisms described to date are activated from this point onwards [59, 151]. Various studies have described the role of genes involved in both chitin

synthesis and its degradation, an aspect to bear in mind regarding this collection of clinical isolates for studying this genotypical component which should lead to better understanding of the pattern observed [11, 119, 152].

Table 12. Phenotypic traits of clinical isolates having reduced susceptibility according to CLSI M27-A3

STRAIN	GROWTH RATE	ADHESION	BIOFILM	COLONY MORPHOLOGY			FLUORESCENCE (CHITIN)
				FCS	SPIDER	SLD + Meth	
SC5314	++	+++	++	Wrinkled	Wrinkled	Peripheral invasion	+
UR608	++++	++++	+++	Wrinkled	Wrinkled	Smooth	++
UR475	+++	+	+	Smooth	Smooth	Smooth	++
UR78	+++	++	+	Smooth	Smooth	Smooth	++
UR147	++++	+	+++	Smooth	Wrinkled	Smooth	++
UR527	+++	+++	+++	Smooth	Smooth	Smooth	+
UR242	++++	+	++	Smooth	Smooth	Slight Peripheral invasion	+

3.6. MALDI-TOF MS characterisation of clinical isolates

MALDI-TOF MS has been validated as a methodology which improves the diagnosis of fungal infection, having 98.5% correct identification for *C. albicans* when the protein extraction protocol is followed by identification involving spectrometry [154, 155]. Classification analysis based on the spectra for clinical isolates, comparing them to those for the reference strains, led to interesting findings regarding the local epidemiology of the hospitals participating in this study, highlighting such statistical strategies' usefulness as a tool for approaching problems concerned with nosocomial infection involving atypical germs.

Identification was made in two phases; the first was taken from a batch of 40 nosocomial samples labelled (UR) from third-level hospitals in Bogotá which were sent to CBS-KNAW in Utrecht, The Netherlands. MALDI-TOF MS output classified 5 of them as *C. albicans*– *C. africana*, 2 of them as *C. glabrata*, 1 of them as *C. tropicalis* and 2 unidentified. These 5 atypical ones were then confirmed in Colombia in duplicate, with at least three biological repeats. Two hundred and forty samples were obtained from the San Ignacio teaching hospital (EZ) and processed during a second phase by MALDI-TOF MS, an extra 6 isolates being classified as *C. albicans*– *C. africana*, following protein extraction in formic acid/ethanol by the University Javeriana's Human Proteomics and Mycosis Research Unit (Bogotá, Colombia).

MALDI TOF MS provided identification with scores >2.0 for all strains, tested in duplicate; analysis included the *Candida albicans* ATCC 90028, SC5314, *C. africana* ATCC 2669 and *Candida dubliniensis* ATCC MYA-646 reference strains and the clinical isolates (**Table 1**). This was the first finding of atypical *C. albicans* isolates in samples collected from different hospitals in Colombia, allowing more elements of analysis to be integrated regarding this fungi's pattern in a hospital setting.

The importance of early identification of Candida species in clinical practice can make the difference for the outcome of the patient, when beginning suitable treatment by searching for the diagnostic method offering the best cost-effective option [156]. Using MALDI TOF MS for characterising this group of clinical isolates provided 98.5% precision regarding *C. albicans* species diagnosis by the protein extraction methodology [155], supporting the fact

that the phenotypical findings described for this group of isolates did indeed correspond to the *C. albicans* species and the parameter for comparison (reference strain SC5314).

Concerning the isolates identified as *C. albicans – C. africana* obtained in samples from patients having the criteria for nosocomial infection, no similar description was found in the pertinent literature, meaning that all the isolates described by other authors came from outpatients' vaginal flow samples and just one blood sample from a patient in Chile [20, 157-161]. Concerning infection epidemiology, the report described in this work highlights the importance of a precise definition of the different types of Candida species which could compromise a patient, given the relevance involved in assigning a specific pathogenicity pattern [20, 162] and avoiding overestimating *C. albicans* as a nosocomial pathogen in this case.

Table 13. Clinical isolates' phenotypical and genotypic characterisation.

Morphology as a phenotypic trait regarding chlamydospore production present in *C. albicans* and *C. dubliniensis.* Conventional mycology tests such as substrate assimilation, growth and growth in chromogenic medium. Molecular markers suggested by international guidelines for panfungal identification and the specific molecular marker *HWP1*. MALDI-TOF MS classification according to Bruker MALDI Biotyper Library.

	Candida albicans	***Candida dubliniensis***	***Atypical Candida albicans***										
	SC5314	**MYA-646**	**CO_B41**	**CO_B44**	**CO_B60**	**CO_B69**	**CO_B77**	**CO_B80**	**CO_R6**	**CO_R41**	**CO_R111**	**CO_R282**	**CO_R425**
Morphology													
Germ tube formation	+	+	+	+	+	+	+	+	+	+	+	+	+
Chlamydospore production	+	+	-	-	-	-	-	-	-	-	-	-	-
Pseudohyphae	+	+	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
Substrate assimilation													
Trehalose	+	+	-	-	-	-	-	-	-	-	-	-	-
Glucosamine	+	+	-	-	-	-	-	-	-	-	-	-	-
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+
API 20C AUX Code	2576174	6152034	2576034	2576034	2576034	2576034	2576034	2576034	2576034	2576034	2576034	2576034	2576034
Growth													
At 30ºC	+	+	+	+	+	+	+	+	+	+	+	+	+
At 37ºC	+	+	+	+	+	+	+	+	+	+	+	+	+
At 42ºC	+	-	-	-	-	-	-	-	-	-	-	-	-
At 45ºC	+	-	-	-	-	-	-	-	-	-	-	-	-
Growth in chromogenic medium													
CHROMagar	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green
Molecular markers													
D1/D2 domains			100% identity with *C. albicans*	100% identity with *C. albicans*	100% identity with *C. albicans*	100% identity with *C. albicans*	100% identity with *C. albicans*	100% identity with *C. albicans*	99% identity with *C. albicans*	100% identity with *C. albicans*	100% identity with *C. albicans*	100% identity with *C. albicans*	100% identity with *C. albicans*
HWP1 gene	940 bp	569 bp	940 bp	940 bp	940 bp	940 bp	940 bp	940 bp	940 bp	940 bp	940 bp	940 bp	940 bp
Bruker MALDI Biotyper Library													
Score > 2.0	*C. albicans*	*C. dubliniensis*	*C. albicans - africana*	*C. albicans - africana*	*C. albicans - africana*	*C. albicans - africana*	*C. albicans - africana*	*C. albicans - africana*	*C. albicans - africana*	*C. albicans - africana*	*C. albicans -africana*	*C. albicans -africana*	*C. albicans -africana*

3.6.1. Atypical isolates revealed by MALDI spectra analysis

Cluster analysis (**Figure 24A**) and MDS (**Figure 24B**) showed four well supported groups, regardless of the clustering method and distance used. *C. dubliniensis* (green) reference strain was clustered as a differentiated element as well as *C. africana* (purple) reference strain. The clinical isolates coded EZ (red) from the San Ignacio hospital were associated with the *C. albicans* strains (blue). The UR isolates (black), collected from five third-level hospitals different to San Ignacio, were grouped in an independent group, but related to the EZ - *C. albicans* group. This represents one of the practical applications for research tools related to clinical practice because, from such methodological approach, the spectra of the isolates were taken for mathematical reading, thus obtaining clustering leading to an initial characterisation of a group or rather a particular population [121, 163]. These findings must be corroborated using carbohydrate assimilation tests and morphological tests, contributing towards defining the species to be described [21].

The subgroups were well differentiated, even when being compared to the reference strains according to proteome identification by MALDI TOF MS. These isolates' nosocomial distribution led to the better identification of new nosocomial atypical species indicating the importance of constructing local epidemiology in each hospital [55].

Using this proteomics tool for characterising nosocomial populations of *C. albicans* represents a great opportunity for constructing local libraries, thereby providing comparison

and evolution parameters regarding these atypical species' nosocomial presentation according to the recommendations in the pertinent literature [55, 164].

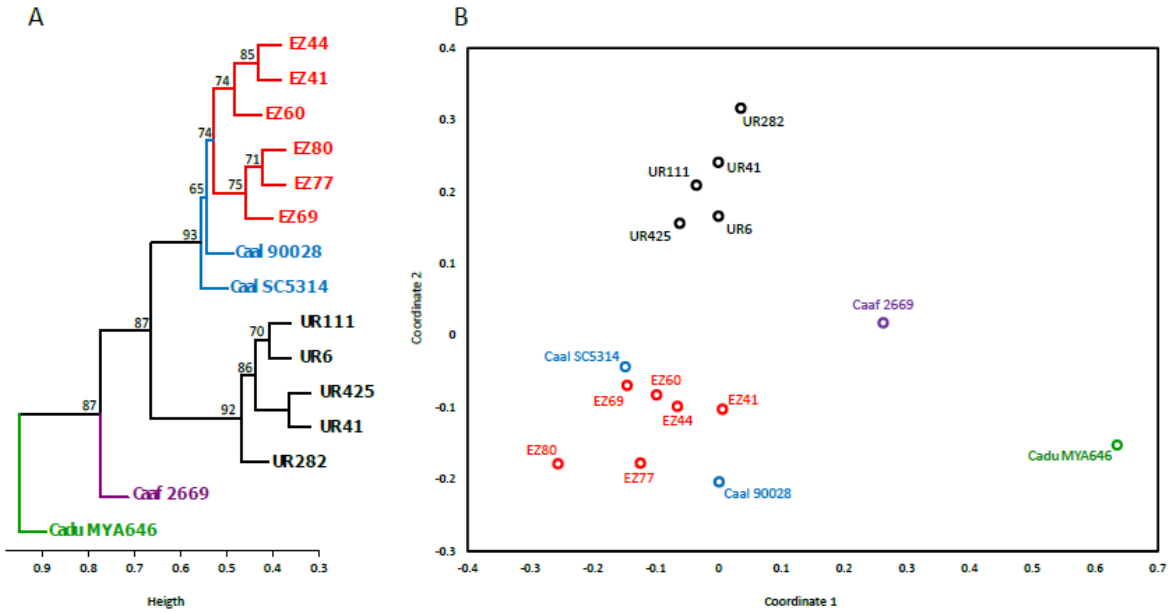


Figure 24. Cluster analysis and metric multi-dimensional scaling (MDS).

A. Cluster analysis. The dendrogram was calculated using UPGMA over absolute correlation distance matrix. Weighted bootstrap values are indicated (> 95 is considered well supported). B. Metric multi-dimensional scaling (MDS). The scatter plot shows the projection of the absolute correlation matrix in two dimensions. Mass signatures of reference spectra (r) from *C. albicans* (strains ATCC 90028, SC5314, in blue), *C. dubliniensis* (ATCC MYA-646 in green) and *C. africana* (ATCC 2669, in purple). Clinical isolates San Ignacio hospital: EZ (in red). Clinical isolates from other hospitals: UR (in black).

3.7. Morphological and physiological characterisation of atypical *C. albicans* isolates

Conventional fungal identification tests were carried out as MALDI-TOF MS revealed atypical *C. albicans* isolates. One such test involved growth in corn meal agar for verifying chlamydospore formation. Chlamydospore formation was not observed in any of the 11 atypical isolates analysed after a 5-day incubation (**Figure 25B**). Three biological repeats were performed. The SC5314 strain was used as positive control, which revealed

chlamydospore formation (**Figure 25A**); Candida *glabrata* ATCC 2001 was used as negative control and did not show chlamydospore formation (**Figure 25C**).

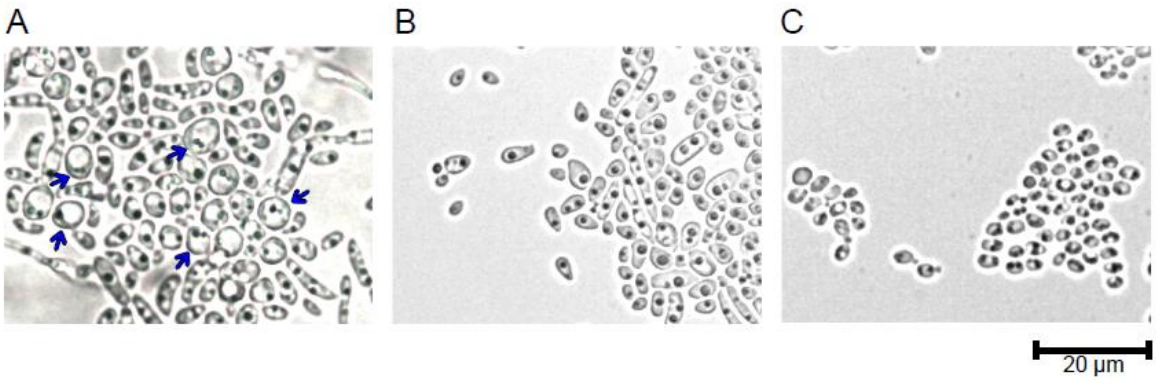


Figure 25. Evaluating chlamydospore production in corn meal agar.

(A). *Candida albicans* SC5314 (positive control), chlamydospores were observed after 5 days incubation (blue arrows). (B). UR6 clinical isolate, no chlamydospores were observed after the same incubation period. (C). *Candida glabrata* ATCC 2001 (negative control), no chlamydospores observed. Image captured by Motic BA200 light microscope (40x).

Regarding carbon assimilation tests, it was found that the eleven clinical isolates did not assimilate trehalose or glucosamine in culture medium (**Figure 26**); moreover, API 20C AUX test results showed that these isolates did not assimilate N-acetyl-glucosamine (like the *C. africana* ATCC 2669 reference strain). By contrast, the *C. albicans* SC5314 and ATCC 90028 control strains were able to assimilate trehalose, glucosamine and N-acetyl-glucosamine. All clinical isolates were unable to grow at 42ºC in growth temperature tests.

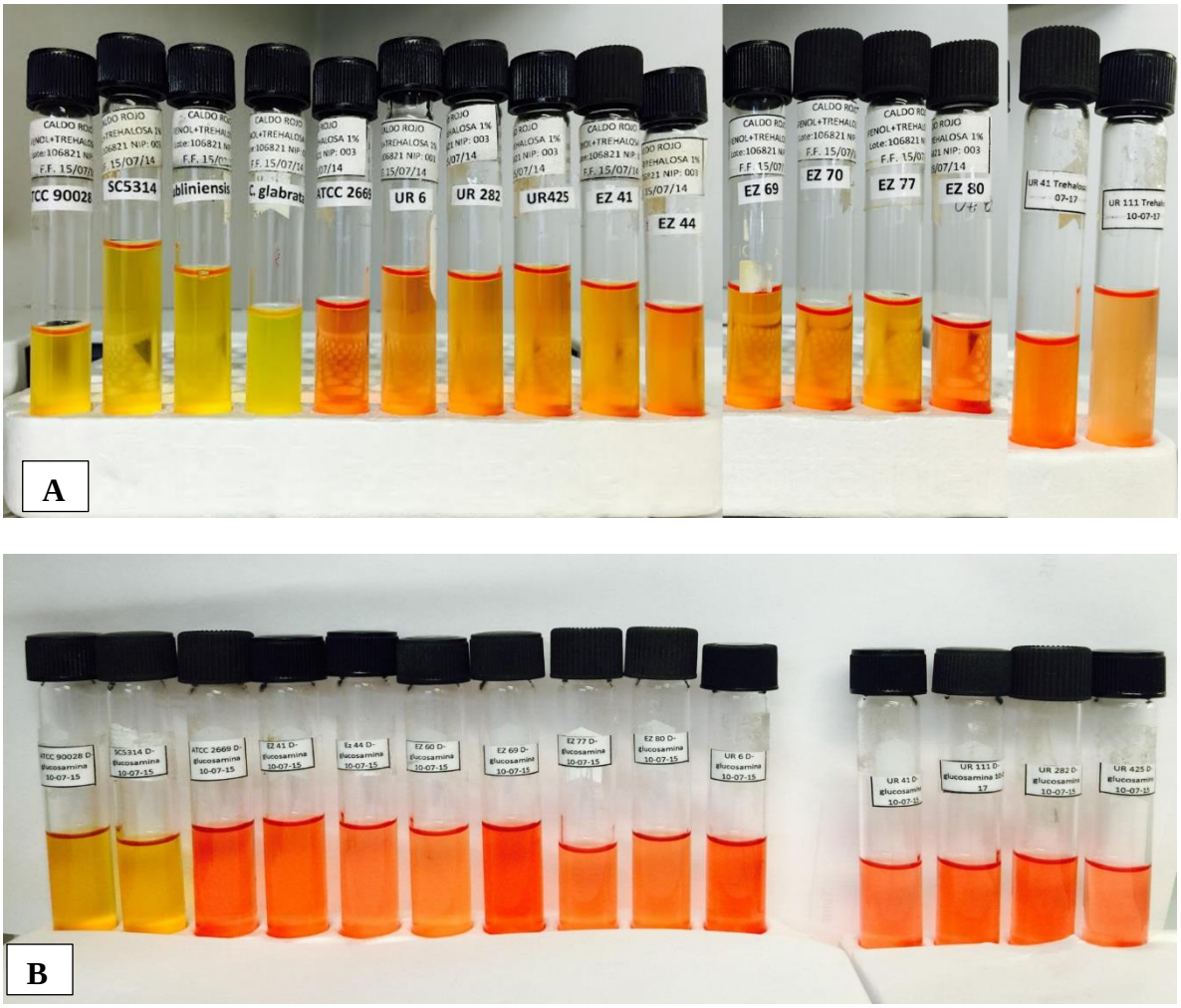


Figure 26. Carbon assimilation tests.

The eleven clinical isolates did not assimilate **A**. Trehalose or **B** D- glucosamine in culture medium. Liquid medium containing trehalose, glucosamine and then sucrose as sole carbon source (5g/L). The amount of yeast inoculated into the medium was 10^3 CFU; this was read after 2 and 5 days incubation at 30°C. Color shift from red to yellow means carbon assimilation. Reference strains: *C. albicans* ATCC90028 and SC5314, *C. dubliniensis* ATCC MYA-646 and *C. glabrata* ATCC2001, all shifted color (Panel A, tubes 1-4 and Panel B, tubes 1-2). No color shift was observed for the reference *C. africana* strain ATCC 2669 (Panel A, tube 5 and Panel B, tube 3).

Table 13 summarises the main findings, comparing the clinical *C. albicans* reference strain SC5314 and *C. dubliniensis* ATCC MYA-646 (as a related strain) to atypical isolates.

Chlamydospore formation can be a morphological trait differentiating *C. albicans* (+ for chlamydospore formation) from *C. africana* (- for chlamydospore formation) (**Table 12**). This criterion has been shown to have physiological relevance since chlamydospore-forming species tend to be resistant to stress conditions [21]. The remaining phenotypical criteria applied to our clinical isolates also agreed with typical *C. africana* characteristics, i.e. inability to assimilate trehalose, glucosamine or N-acetyl-glucosamine [21]. According to Tietz *et al*., [21] these morphological and physiological characteristics are considered a very important taxonomic criterion which would lead to our atypical isolates being identified as *C. africana* morphospecies.

An inability to assimilate N-acetylglucosamine and glucosamine amino sugars explains the inability to form chlamydospores, according to the pertinent literature [21]. Pedreño *et al.*, (2004) have shown that cell wall acid trehalase (Atc1p) activity is compromised as trehalose is not assimilated [165]; this enzyme metabolises trehalose as carbohydrate source in the absence of glucose or other carbohydrates.

An article was published stating that this enzyme is involved in the transformation of yeast to hypha in *C. albicans* and it has also been described that its activity increases in the presence of cell wall permeabilisation agents, such as toluene/ethanol/Triton (TET) [166]. Carbohydrate assimilation tests for *C. albicans* atypical species have revealed evolutionary adaptations or changes related to the cell wall pattern of this fungus.

Regarding the lack of these atypical isolates' growth at 42ºC, Maidan *et al.,* (2008) showed how the growth curve descended to 43ºC in mutated laboratory strains having a *GPR1* gene

allele deleted (involved in signalling and hyphae formation in the presence of methionine) and complete deletion of the *TPS2* gene (involved in trehalose synthesis as protection factor regarding cell stress). They also observed that these strains were thermolabile compared to the reference strain in a further test after submitting mutated strains to temperature stress by incubating them at 44ºC for periods of 0 to 3 hours and then letting them grow at 30ºC in YPD [115].

The above findings suggested that *C. albicans* atypical clinical isolates have significant differences regarding their physiological and morphological pattern related to proteins and enzymes located in the fungal cell wall. Further studies are required for elucidating the pattern of genes and products modelling the phenotypical plasticity observed regarding these microorganisms' virulence and susceptibility.

3.8. Molecular characterisation of atypical *C. albicans* isolates

Concerning molecular markers, D1/D2 sequences from the eleven isolates had ≥99% sequence identity with *C. albicans* (**Table 13**). Regarding the *HWP1* gene amplification, all clinical isolates had the ~940 bp band characteristic of *C. albicans*, whilst the *C. africana* ATCC 2669 had the expected ~ 700bp amplicon and *C. dubliniensis* control strain ATCC MYA-646 displayed the expected ~569 bp amplicon (**Figure 27**).

Clinical isolates were identified differently when molecular markers were used; according to the D1/D2 sequencing, all clinical isolates were *C. albicans*. However, several authors have stated that the small difference in sequence (just 1%) between *C. albicans* and *C. africana*

obtained using this molecular marker does not properly differentiate between these two species [157, 167]. The *HWP1* gene was thus used to overcome this problem, which has been reported to be appropriate for differentiating *C. albicans, C. africana* and *C. dubliniensis* [125]; this marker also identified our 11 atypical isolates as *C. albicans* (**Table 13**).

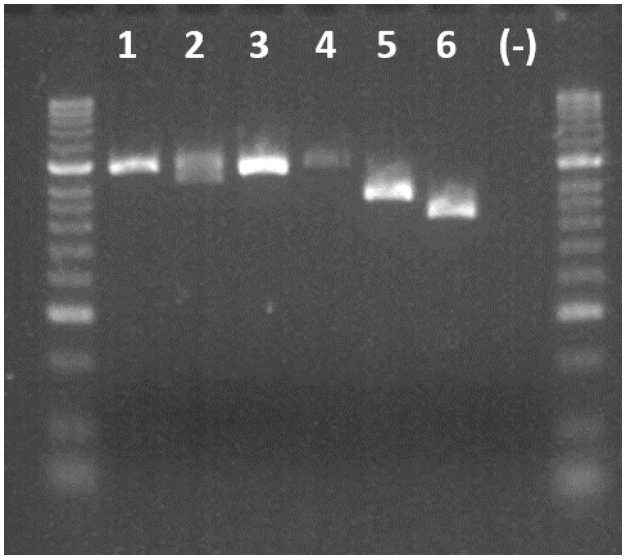


Figure 27. *HWP1* amplification.

Clinical isolates 1: UR111 and 2: EZ44 and Reference strains 3: SC5314; 4: *C. albicans* ATCC90028; 5: *C. africana*; 6: *C. dubliniensis*. The expected amplification products were observed for reference strains: 941 bp for *C. albicans*, 700 bp for *C. africana* and 569 bp for *C. dubliniensis*. A 941 bp amplification product was observed for the 3 clinical strains.

HWP1 gene analysis has been proposed as being a rapid molecular technique for differentiating *C. albicans, C. africana* and *C. dubliniensis*; in a report of a clinical case in Italy, the amplification product obtained from this isolate had equal size to that found for the *C. albicans* reference strain, but different to that from *C. dubliniensis*, its closely-related species, even though other findings using conventional identification agreed with *C. africana* typing (**Figure 27**) [160]. Moreover, *HWP1* molecular identification was performed

according to Romeo *et al*., 2008 [125] and the expected products (as mentioned beforehand) identified our clinical atypical isolates as *C. albicans*. Such findings, supported Romeo and Criseo's notion that this is a useful marker; however, additional methodologies are needed for atypical isolates to differentiate the species belonging to this complex [19]. By contrast, the conventional mycological procedures (carbon assimilation test, chlamydospore formation, ability to growth at 42ºC), would indicate that these isolates belong to *C. africana*.

Such findings question the taxonomic position of these fungi. Some authors consider *C. albicans* as a species complex consisting of related species, such as *C. africana*, *C. stellatoidea* and minor species such as *C. dubliniensis*, always related to some clinical particularities associated with species which, regarding *C. africana*, are associated with the presentation of vaginal infection, and in the case of *C. dubliniensis*, a preceding compromise by HIV and oral localisation, and seems more prevalent in cystic fibrosis patients [20, 24, 25]. *C. africana* infection has been described worldwide in Senegal, Madagascar, Nigeria, Angola, Germany, Italy, the UK, Spain, Saudi Arabia, India, Japan, the USA, Chile and China [20, 157-159, 161, 168, 169].

Regarding the current work, it has been the first to describe nosocomial isolates having such classification as atypical *C. albicans* as their morphological and substrate assimilation fit with *C. africana* and, concerning the clinical data reported here, that only one isolate came from vaginal infection whilst the remaining isolates came from urine, blood, faeces or bronchoalveolar lavage (**Table 14**).

Table 14. Clinical information regarding the atypical isolates studied.

Isolate	Patient gender	Age (in years)	Source	AF treatment	Clinical outcome	Clinical setting	Locality
UR6	F	55	Urine	None	Dead	ICU	Kennedy
UR41	M	1 month	Blood	Amphotericin B	Alive	ICU	Usaquen
UR111	M	18	Urine	Fluconazole	Alive	ICU	Tunjuelito
UR282	F	70	Urine	None	Dead	Room	San Cristobal
UR425	M	70	Urine	None	Dead	ICU	Kennedy
EZ41	M	44	BAL	Fluconazole	Alive	Room	Chapinero
EZ44	F	26	Vaginal swab	N.D.	N.D.	N.D.	Chapinero
EZ69	F	68	Faeces	None	Alive	Room	Chapinero
EZ60	M	94	Urine	None	Alive	Room	Chapinero
EZ77	M	18	BAL	None	Alive	Room	Chapinero
EZ80	F	73	BAL	Voriconazole, caspofungin, amphotericin B	Dead	Room	Chapinero

BAL: bronchoalveolar lavage; N.D: No data.

3.8.1. Gene expression in an atypical isolate

One of the clinical isolates whose phenotypical classification corresponded to a denomination of atypical *C. albicans* was selected in the group of clinical isolates considered for the expression tests in the genes of interest related to cell wall stability as a response mechanism to stress caused by caspofungin. Initial selection was based on observing that this atypical *C. albicans* led to the death of the patient carrying it as outcome, as well as having an intermediate susceptibility pattern (0.5 µg/mL) in the susceptibility test according to CLSI M27-A3, i.e. tolerance to exposure to caspofungin.

The expression pattern for the genes of interest was as follows. The *FKS1* gene had significant upregulated expression (**Figure 16**), surpassing that in the *C. albicans* SC5314 reference strain as well as in the other clinical isolates identified as *C. albicans.* Being an atypical *C. albicans* strain, this result indicated that cell stress caused by caspofungin acted on this gene's expression associated with the Rho regulatory subunit implicated in activating the PKC pathway, thereby increasing chitin production in the cell wall as compensation mechanism [59]. Bearing in mind that caspofungin's mechanism of action is the non-competitive inhibition of the Fks1 protein [60], then such overexpression pattern shows compensation action against antifungal activity. Reviewing the pattern for the *C. albicans* reference strain SC5314 revealed a different situation as it involved slight down-regulation.

Regarding the *CNB1* gene, there was greater upregulation in this atypical isolate than in clinical reference strain SC5314 and in *C. albicans* clinical isolates (**Figure 17**). The

pertinent literature states that this gene forms part of the calcineurin pathway and this pattern could be associated with a compensation mechanism interacting with the *FKS1* gene [59, 72].

Figure 16 shows outstanding *ERG5* gene expression in the atypical *C. albicans* UR282 isolate; the pertinent literature describes this gene's expression in laboratory strains resistant to fluconazole and amphotericin B [149]. It has also been described that exposure to azoles affects cell wall integrity [16]; caspofungin also alters cell wall integrity due to its mechanism of action [14]. This could explain the significance of the *ERG5* gene's overexpression in UR282. The foregoing is interpreted as a cell wall stability compensation mechanism given the susceptibility pattern observed. However, additional studies are needed for identifying precisely the link described between exposure to caspofungin and the overexpression of this gene involved in the cell membrane integrity.

Reviewing *PGA13* gene expression in clinical isolate UR282, it was found that this gene was significantly overexpressed on being exposed to caspofungin when compared to expression in reference strain SC5314 and *C. albicans* clinical isolates (**Figure 18**). *PGA13* gene overexpression showed how exposure to caspofungin activated the cell wall regulation pathway, mediated by transcription factor *CAS5* responding to damage to the cell wall which caspofungin can cause [37]. It has been described that this factor induces filament formation in SPIDER growth-inducing medium [170], thereby confirming results from our filamentation assays for this strain (**Figure 28**). Regarding activating the cell wall regulation (CWR) pathway, work by Pukkila-Worley *et al.,* (2009) has elegantly shown how complete deletion of *CAS5* attenuated *C. albicans* virulence in the nematode *Caenorhabditis elegans* [170].

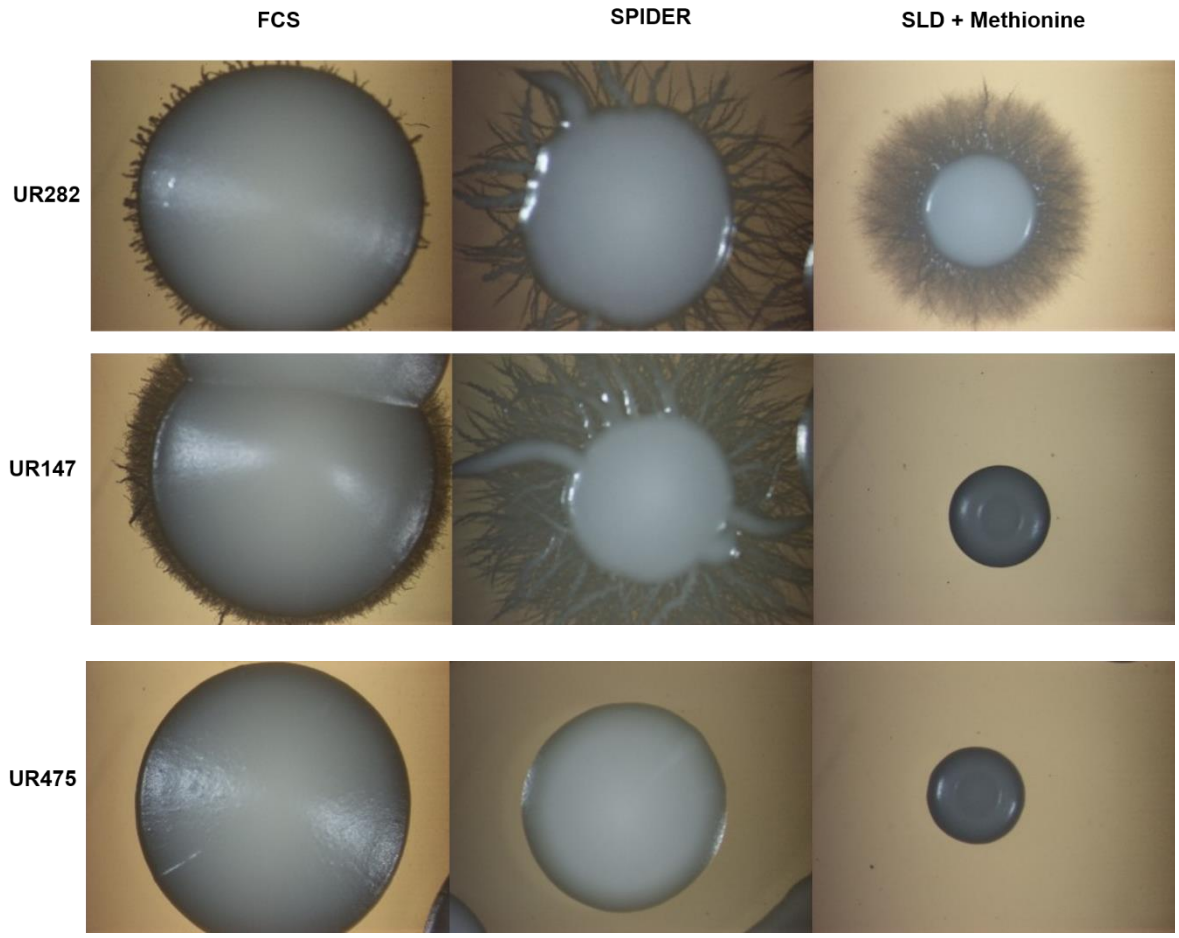


Figure 28. Colony morphology.

Atypical *C. albicans* isolate UR282 compared to typical *C. albicans* isolates UR147 and UR475. FCS: Foetal calf serum, SPIDER media and SLD media supplemented with methionine.

PGA62 encoded protein is a GPI-anchored product involved in *C. albicans* cell wall structure and significant overregulation was observed in this expression assay compared to reference strain SC5314 (**Figure 18**). Plaine *et al.*, (2008) have shown how this GPI-anchored protein is involved in cell wall integrity and that the mutant laboratory strain having double deletion in the *PGA62* gene has hypersensitivity to Calcofluor White (CFW), an agent causing cell wall stress and resistance to caspofungin [36].

Continuing to note proteins involved in cell wall stability concerning stress factors, the *PGA31* gene is significantly overexpressed (**Figure 19**) and the aforementioned work by Plaine *et al*., (2008) shows how the mutated strain having double deletion of this gene is hypersensitive to CFW and highly susceptible to caspofungin [36], i.e. significant overexpression of *PGA31* effectively protects a cell from stress caused by exposure to caspofungin. Chitin content in the cell wall becomes significantly reduced in the mutated strain having double deletion following the pattern of overexpression; this could be interpreted as a compensation mechanism increasing cell wall chitin content.

Png2 is one of the proteins externally located in the cell wall which is implicated in *C. albicans* adhesion and adaptation; its *PNG2* gene [150] was overexpressed in isolate UR282 compared to reference strain SC5314 in this assay (**Figure 19**). Such pattern agreed with that stated in the pertinent literature describing exposure to caspofungin as increasing this gene's activity, also being associated with reduced susceptibility to caspofungin, this being one of the results obtained in the present work [37, 149].

Exploring response pathways guaranteeing cell wall stability revealed that exposure to caspofungin activates the PKC pathway [37]; this coincided with our expression assay where significant *MKC1* gene overexpression in isolate UR282 was observed (considered atypical *C. albicans*) and in clinical isolate UR147 (considered typical *C. albicans*) (**Figure 20**). The pertinent literature states that activating the PKC-MAPK-dependent pathway is related to the induction of filamentation [140]; this was shown in filamentation tests on the strains in the study where results for UR282 and UR147 again agreed with such finding (**Figure 28**).

Another coincident aspect concerned the outcome of death in patients infected by these isolates.

The changes in expression observed in atypical *C. albicans* isolates revealed clear differences regarding reference *C. albicans* and *C. albicans* from clinical isolates, thereby providing valuable information for continuing research into the evolutionary mechanisms facilitating the adaptation of this variety of *C. albicans* in the search for opportune therapeutic approaches.

Chapter V - Association between clinical risk factors, phenotypic traits and the presence of reduced susceptibility to echinocandins in nosocomial strains of *Candida albicans*.

Regarding susceptibility patterns and findings related to the clinical isolates' phenotypical traits, the distribution of nosocomial *C. albicans* infection showed that 7 clinical isolates (13%) came from non-ICU ward discharges and 24 clinical isolates (87%) from ICUs. **Table 15** gives the related clinical characteristics. No significant differences were found regarding gender distribution (51.6% females and 48.4% males; p=1.00, exact binomial test). In age groups known as being at risk of acquiring infection, 8 patients (26%) aged less than 1 year old and 7 patients (22%) older than sixty were found. There was 33% mortality, similar to that described in the literature [5].

Diagnosis for nosocomial infection caused by *Candida* spp. had a similar distribution between invasive candidiasis (58.1%) and urinary tract infection (41.9%) caused by *C. albicans*. Regarding risk factors, more than four broad-spectrum antibiotics were being used at the same time (10 patients: 32.3%), central venous catheter use (25 patients: 80.6%), parenteral nutrition (18 patients: 58.1%) and more than 15 days' hospital length of stay (27 patients: 87%).

Table 15. Demographic and clinical characteristics

Variable	ICU n cases (%)	Non-ICU ward n cases (%)
Age ≤ 1 year	7 (23%)	1 (3%)
Age ≥ 60 years	5 (16%)	2 (6%)
Male gender	13 (42%)	2 (6%)
Invasive Candidiasis	14 (45%)	4 (13%)
UTI	10 (32%)	3 (10%)
Mortality	7 (23%)	3 (10%)
Broad spectrum AB use	24 (77%)	6 (19%)
CVC	21 (68%)	4 (13%)
Abdominal surgery	6 (19%)	4 (13%)
TPN	15 (48%)	3 (10%)
Immunosuppressant	6 (19%)	0
Length of stay ≥15 days	22 (71%)	5 (16%)

Note: ICU: intensive care unit, UTI: urinary tract infection. AB: antibiotic, CVC: central venous catheter, TPN: total parenteral nutrition.

4.1. The clinical association between patients' risk factors and reduced susceptibility to echinocandins

Bivariate analysis revealed an association between fluconazole resistance and type of hospital, greater resistance being found in private (20%) than public hospitals (0%) (Fisher's exact test p=0.09) and equal results were found regarding multi-resistance concerning fluconazole and caspofungin (Fisher's exact test p=0.09).

Regarding risk factors and an association with resistance to caspofungin, resistance in patients without abdominal surgery was significantly greater (33.3%, 4/12, *cf* 5.3%, 1/19, p=0.021, Mann Whitney exact test).

There was a significant association between mortality and low socioeconomic level, mortality being greater in patients having a daily income of less than 9€ (50.0%, 9/18 *cf* 15.4%, 2/13) (p=0.05, Fisher's exact test). *Candida albicans* infected patients with no parenteral nutrition had a significant association with mortality when compared to those having parenteral nutrition (61.8%, 8/13 *cf* 16.7%, 3/18) (p=0.014, Fisher's exact test).

The multivariate exact unconditional logistical regression model for mortality was significantly associated with the absence of parenteral nutrition (OR=39.746: 1.794- 880.593 95%CI; p=0.020) and closely associated with a background of being undernourished (p=0.079, OR=14.21). The other factors were not significant (having an abdominal surgery during hospital stay, the number of antibiotics or length of hospital stay). The model's discrimination power was 83.9% (85.9% area under the ROC curve: 69.5-100 95%CI; p=0.001).

No statistically significant associations were found between the susceptibility pattern and the clinical outcomes.

It is worth noting that this is the first observational study reported in a population having a higher nosocomial fungal infection rate (12.2/10,000 discharges) than that reported in pertinent literature (4.9/10,000 discharges [171]) which highlights the importance of the results here found, despite the relatively low sample size. However, associations were established using exact non-parametric statistical tests having a high statistical power

(>95%), suitable for small sample sizes [172, 173] to overcome this problem, relevant aspect concerning studying *C. albicans* infection concerns the clinical factors associated with a risk of contracting the infection [49]. In spite of some reports having indicated an increase in infections caused by non-albicans species, *C. albicans* continues being responsible for most cases involving fungal infections in hospitals [40], besides, the associated risk factors have not changed. The overall description of patients suffering *C. albicans* infection in our study has shown that the presence of central venous catheter, prior broad spectrum antibiotic use and parenteral nutrition had the greatest association [174], as well as most patients having stayed in an ICU.

Statistical analysis of 31 patients having nosocomial *C. albicans* infection identified significant associations which were related to the two most relevant variables: mortality and nutritional state.

The study revealed an association between patients having a daily income of less than 9€ and mortality which was not comparable with that stated in the pertinent literature. This factor is an external agent affecting patient care over which the healthcare team has no control and is associated with their socioeconomic level. However, factors were explored which were related to the type of hospital or haemodynamic state and which might have affected this variable; no significant values were found, thereby indicating that the patients' level of medical treatment was equitable for all patients.

Regarding bivariate analysis of patients infected by *C. albicans*, another strongly associated factor referred to the absence of parenteral nutrition and mortality when contrasting our study with the literature related to mortality studies. This factor was not specifically mentioned [5], though references were made to the presence of parenteral nutrition and a risk of candidaemia. However, some important work refers to nutritional state and infection and how compromise regarding nutritional state favours the risk of infection and even a patient becoming immunocompromised [175]. Multivariate analysis covering all the risk factors for mortality and candidaemia described in different work [5, 176] was taken into account when designing an exact unconditional logistical regression multivariate model which led to finding a significant association between the absence of parenteral nutrition. This indicated the relevance of including patients' nutritional state and the relevance of including suitable nutritional support for providing their needs in a clinical evaluation.

Several studies have been published about nosocomial *Candida* spp. infection presentation mode and frequency, as well as regarding the risk factors associated with the infection [5, 176]; however, little information is available regarding patients' susceptibility, socioeconomic conditions and nutritional support. The nosocomial infection rate found in the present study was representative of the samples taken in the tertiary care hospitals which participated in the study and was even higher than that reported in the literature [177]. The relevance of these results has emphasised aspects regarding prevention and the rational use of antifungal therapeutic schemes, expressed by other authors as being one of the best strategies for controlling this emergent problem [178, 179].

4.2. The clinical association between patients and atypical *Candida albicans* isolates

The samples were collected in six third-level general hospitals in Bogotá, Colombia. Samples labelled as CO_B were isolated from clinical settings involving one teaching hospital (San Ignacio); those labelled CO_R came from 5 third-level general hospitals and most of them (4/5) were isolated from intensive care units (ICU). The clinical information related to the samples revealed that only one of them involved vaginal flow; the remainder came from urine, bronchoalveolar lavage or blood. There were no significant differences regarding the gender of these 11 patients and half of them were more than 60 years-old. Four of them had received antifungal treatment associated with a diagnosis of *Candida* infection. **Table 14** shows that, according to each patient's clinical condition (i.e. urinary tract infection, invasive candidiasis and candidaemia) and following international protocols [62], patients received one or several of the following antifungals in the doses shown: fluconazole (400-800mg/day), voriconazole (6/3/mg/kg/day), caspofungin (70/50mg) or amphotericin B (0.7-1.0mg/kg). Even though this was a small sample, it should be stated that four out of this group of 11 patients died and one of these patients died after having received sequential treatment involving voriconazole, caspofungin and amphotericin B due to invasive candidiasis. All patients had an underlying condition; this was cancer-related in two cases, one had hepatic failure and another one suffered chronic renal failure (**Table 14**).

Regarding the origin of the clinical isolates in this work, it is worth highlighting that only one came from the vagina, the rest from different anatomical locations (**Table 14**), differently to that described in the literature concerning the *C. africana* species [20, 21]. This means that the spectrum of infection was broader in this report and was even associated with triggering

the death of some patients (4/11); however, the underlying conditions pertaining to comorbidity should be also taken into account. The small sample size did not lead to accurately attributing the mortality rate, but indicates the need for further studies involving an active search for cases to better establish the degree of impact represented by these atypical species [20].

Clustering and MDS results revealed well-defined differences amongst *C. albicans*, *C. africana* and *C. dubliniensis* reference strains and, more interestingly, two groups of clinical isolates, one formed by EZ isolates, clearly related to *C. albicans* reference strains and another formed by UR samples. Two different forces modelling the clustering pattern in clinical isolates may thus be proposed. Place of origin and ICU background (related to prophylactic antifungal therapy) can drive these pathogens' local epidemiological landscape. UR isolates came from different hospitals, but had a common background related to critical patients' conditions; 4 of the 5 were hospitalised in an ICU and 3 of the 5 died in this group. According to ICU protocols or these patients' critical condition, previous exposure to fluconazole could be assumed in these nosocomial isolates (**Table 14**). Otherwise, EZ isolates from a clinical setting involving only one hospital had the same place of origin as a common characteristic; these isolates came from patients who had not been in an ICU, so this group of patients did not have a critical condition related to prophylactic antifungal therapy. Consequently, the grouping observed for the isolates from both groups might have reflected differential selective drug pressure and origin.

Chapter VI - Conclusions

This thesis' main interest lay in ascertaining the role of the *FKS1* gene concerning the susceptibility and virulence of a collection of nosocomial *C. albicans* clinical isolates from 10 third-level hospitals in Bogotá, Colombia.

The CLSI M27-A3 methodology was initially used regarding susceptibility patterns [96]; caspofungin was the antimycotic initially selected for this screening from a significant percentage (13%) of clinical isolates which showed reduced susceptibility. However, the susceptibility patterns were adjusted according to the pertinent literature following a CLSI review regarding the difficulty of reproducing caspofungin susceptibility values in international reference laboratories, recommending a change of guidelines to CLSI M27-S4 [98]. The number of isolates having reduced susceptibility to micafungin became substantially reduced to just one by changing the methodology; however, as shown in the different assays used when developing our research objectives, information initially obtained with CLSI M27-A3 methodology was useful when exploring the expression of the genes of interest in nosocomial isolates which initially showed reduced susceptibility to caspofungin using this protocol. Differential expression was found in the group of selected genes, as well as the phenotypical changes which could explain this feature.

Another element highlighted in developing the first objective referring to the absence of mutations in the sites described for the *FKS1* gene described as Hot spot 1 and Hot spot 2 in the clinical isolates showing reduced susceptibility in both CLSI methodologies [15, 59, 180]. This result agreed with several authors' hypothesis posed in work in laboratory strains

[36, 129] as a similar pattern was observed in clinical isolate UR608, where compensation mechanisms for guaranteeing cell wall stability provided *C. albicans* with a degree of tolerance to exposure to echinocandins, thereby promoting resistance [11, 119].

A second aspect born in mind when describing this pattern in a nosocomial isolate was that no prior exposure to caspofungin or other echinocandin was identified from the clinical records for the patient carrying this fungus. The emergent hypothesis after reviewing the literature thus stated that the complex cell wall stability mechanism would become affected after exposure to azoles [16]. Pressure resulting from antifungal use [55] best explained this result, considering the isolate's nosocomial origin as, azole use was more frequent in the hospitals analysed as recommended by their management protocols [62]. Additionally, this isolate's cell wall chitin content was greater than that in the SC5314 clinical reference strain when exposed to a sublethal concentration of caspofungin.

It is worth highlighting the sample size; there were 77,763 regular hospital discharges in non-ICU ward and 5,204 ICU discharges; the ICUs were mixed purpose (i.e. caring for surgical, coronary, septic and poly-traumatised patients). They were selected during a 6-month period when only isolates were taken (101), 30 of which were identified as *C. albicans*. Epidemiological interest in characterising them arose as they complied with the criteria of nosocomial infection [51] thus placing these fungi in a distinct category on being exposed to selective pressure by nosocomial flora mediated by the use of antifungals in the hospitals [53, 55].

The most valuable conclusions from characterising susceptibility patterns firstly concerned CLSI M27-S4 methodology usefulness in defining *C. albicans* nosocomial flora susceptibility patterns. It is worth stressing that the break-points have been cut from ≥2μg/mL to ≥0.5 μg/mL in the seven years in which this methodology has been established, thereby indicating this fungus' dynamic pattern regarding antifungal use. The second conclusion concerned the relevance of the mechanisms responding to cell wall stress showing changes in susceptibility patterns without the echinocandin target gene (*FKS1*) being mutated and without being necessarily exposed to echinocandins, as in our case.

The phenotypical characterisation of the clinical isolates revealed a series of interesting results. The first referred to isolate growth rate compared to that of the clinical reference strain, even though sufficient information could not be found in the pertinent literature for discussing this finding. Brauer *et al.,* (2008) referred to cell stress conditions related to increased yeast growth rate [134]; this aspect merits further research related to this phenotypical feature and mutation rate or speed in adapting to the setting presented by this type of isolate. The filamentation pattern also merits special discussion as most clinical isolates had a contrary pattern to that observed in the reference strains as, according to the literature, filamentation is a virulence criterion [115, 170]. However, filamentation was deficient in clinical isolates associated with the outcome regarding patient mortality. Further studies are thus required for determining this feature's participation in nosocomial isolates.

The expression of the genes of interest related to cell wall stability in the initial screening revealed differential expression in three selected clinical isolates compared to clinical reference strain SC5314. The first aspect to consider, according to the results in the assay

where clinical isolates were submitted to a sublethal concentration of caspofungin, was related to the *FKS1* gene's role in such interaction involving response to the cell wall, as exposure to caspofungin produced changes in the expression of the genes selected for *C. albicans.* Several authors have proposed stability activating responses for the cell wall from stress arising from its disruption where one of the mechanisms deals with *FKS1* gene interaction [37]; this would involve the Fks1 protein which is the subcatalytic unit of the enzyme complex synthesising β1-3 D glucan which binds to the regulatory unit, the Rho protein. This, in turn, is implicated in activating the PKC pathway (one of the main pathways implicated in guaranteeing cell wall stability) when interacting with Wsc1 and Mid2 stress receptors on the cell wall [59].

One of the main discussion points when reviewing antifungal resistance refers to cell fitness implicating an adaptative response [181], resulting in less virulence; however, when describing therapeutic failure in echinocandin-resistant strains [182], interpretation of fitness must be reconsidered regarding the clinical management of a patient infected by a fungus of this type. The changes detected in the expression of genes involved in cell wall stability showed that selective pressure arising from antifungal use [55] merits further research, in addition to considering changes in *FKS1* gene expression detected in nosocomial isolates; this should be studied in depth for understanding the communication networks governing cell wall stability [14] for better treatment of patients involving combined therapies from the start of infection.

A qualitative exploration of cell wall chitin concentration when nosocomial isolates were exposed to a sublethal concentration of caspofungin followed, showing that chitin

concentration was greater in nosocomial isolates compared to in the reference strain. This result was supported in the pertinent literature referring to increased chitin concentration conferred echinocandin tolerance–resistance as compensatory response mechanism providing cell wall stability [119, 151, 152]. It is worth noting that the descriptions reported in the literature to date have been about laboratory strains (i.e. nosocomial isolates in this work), underlining the importance of the study in terms of characterising these clinical isolates due to the implications for patient management.

The work's second objective involved using MALDITOF-MS for identifying the set of nosocomial isolates; 5 clinical isolates reported as *C. albicans – africana* were found in the first lot followed by 6 *C. albicans–africana* isolates in a second lot. This finding merited additional molecular, morphological and physiological identification tests, finding that the isolates in question were *C. albicans* from the molecular point of view [124, 125] and *C. africana* regarding morphological and physiological aspects [21]. These results highlighted the epidemiological importance of characterising epidemiologically significant pathogens at nosocomial level [53] as this avoids overestimating *C. albicans* in a critical disease such as candidiasis or candidaemia [20] as well as advancing the description of and knowledge regarding this atypical species' pathological implications [162, 183]. Tools having great discrimination power and practical usefulness in clinical diagnosis were used for identifying and characterising these isolates, i.e. MALDI TOF–MS [156]; this was combined with MDS analysis of spectra facilitating better clustering of clinical isolates which, according to the pertinent literature, promotes the construction of local epidemiology [164] in each hospital as well as constructing local data libraries improving ability to identify species in nosocomial flora.

One of the atypical strains was selected for the gene expression assay; key differences in *FKS1* gene upregulation were found, as well as that of the genes involved in cell wall stability related to the calcineurin, GPI and MAPK pathways. Interpreting these findings led to grouping involving the following aspects. The strain analysed had significant *FKS1* gene overregulation, indicating that the stimulus produced by caspofungin could be produced by a compensatory response in the activity of the Rho protein. This protein's regulatory subunit has been seen to be involved in activating the PKC pathway where the Mkc1 protein plays a significant role; significant encoding gene upregulation has been observed [59, 133]. The *PGA13* and *PNG2* genes involved in the effector pathway of the Cas5 protein sensitive to caspofungin activity have been highlighted in a second group of key GPI proteins involved in cell wall stability [37]. The *CNB1* and *ERG5* genes both have significant upregulation associated with cell membrane stability when stimulated by azoles; however, differential expression was observed when stimulated by caspofungin highlighting the complex network stabilising cell wall structure [149]. Even though these results come from a first round of assays, their correspondence with that stated in the relevant literature indicates the importance of further research for evaluating the genes involved in cell wall stability.

The main conclusions concerning nosocomial isolate phenotyping refer to the importance of findings regarding growth rate, filamentation ability, the differential expression of genes related to cell wall stability and the identification of atypical *C. albicans* species. This series of phenotypical features indicated a very specialised level of complexity regarding control of cell stress in *C. albicans* cell wall suggesting great plasticity in adaptation mechanisms which could alter conventional identification tests or trace phenotype transition to genotype due to evolution regarding antifungals' selective pressure [137].

It was observed that the *FKS1* gene was present in most events involving response to caspofungin stimulus due to the expression of the genes so detected, as well as increased chitin in the cell wall. Its key role in the regulatory cycle of susceptibility to drugs blocking it led to thinking that combined management regarding this group of antifungals would be desirable; this situation could be shown by the mobility of the clinical breakpoint for susceptibility from ≥2μg/mL to ≥0.5 μg/mL in 7 years in one of the standard tests determining this fungus' resistance to echinocandins.

The implications of the work's second objective are key in developing a methodology for the epidemiological characterisation of nosocomial *C. albicans*, the practical use of tools related to microbiology, molecular biology, proteomics and biomathematics applied to clustering and selecting atypical strains in a hospital context as well as identifying the role of phenotypical expression in these clinical isolates represented by the *FKS1* gene. Work centred on the first group of genes selected where changes were observed in their expression pattern for reconstructing the sequence of events determining phenotypical changes making these clinical isolates change their echinocandin susceptibility pattern. It is hoped that such research will provide more information in the long term towards developing effective therapeutic strategies leading to this infection being treated better.

The clinical aspects of the work determining the work's third objective provide relevant information for clinical practice regarding *C. albicans* infection. A significant association between mortality and malnutrition in this group of patients was revealed in epidemiological and statistical analysis, as well as an association between mortality and reduced economic income. Regarding the first association, some work refers to the importance of nutrition [175]

in managing infection; however, the pertinent literature does not mention this variable as one of the factors to bear in mind when evaluating its participation [5, 176]. No significant differences were found regarding the factor at social level in multivariate analysis concerning patient attention in public and/or private hospitals, indicating that patients were being offered an equitable level of attention. An external factor which is difficult to modify by the healthcare team attending a patient is the aspect concerning low daily income of <9€ daily for this group of patients having a significant association with mortality. All the other associations described as risk factors were analysed without finding statistically significant differences; however, more studies are required involving increased sample size for ascertaining more relevant associations regarding this disease.

Regarding atypical *C. albicans*, this is the first description in Colombia and it is worth noting that its location varied in the group of patients identified with this infection, unlike most typical descriptions isolating Candida from the vagina. According to Tietz *et al.*, (2001) another of the relevant aspects in this group of patients classified in MDS analysis as UR is related to the place where it might occur in a hospital (80% in an ICU) and that 60% die with adjacent comorbidities such as renal disease and cancer. Regarding this pattern, the present work did not give the usual clinical description if this group of isolates was taken as being *C. africana*, meaning that this group must be exhaustively typed and an active search made by epidemiological surveillance of more samples from equivalent conditions for determining clinical patterns with a larger sample size. However, one of the reasons for these isolates grouping was due to the selective pressure of the antifungals in hospitals, despite them coming from different hospitals [55].

The main conclusions related to the development of the third objective were associated with a patient's nutritional condition which could become modified after being admitted to hospital with nutritional support and evaluation (if required) [175]. Regarding the considerations associated with atypical *C. albicans*, MDS associated their grouping with a background of intensive care which should motivate a healthcare team to search for monitoring strategies and the rational use of antifungals, monitoring susceptibility patterns and resistance, besides ascertaining the local epidemiology of infection as the potential damage represented by these atypical species remains unknown at present [184].

The contribution made by developing this third objective was immense given the socioeconomic conditions where the association of mortality and malnutrition was evident where the best message to healthcare teams from these results is not to neglect this aspect regarding our patients as, unfortunately, there is not much a hospital can do to improve patient socioeconomic state, this being a preponderant external factor in associating our patients with a failed outcome. Another key contribution has been the identification of atypical *C. albicans* an extremely well-defined clustering pattern associated with a failed outcome, thereby meriting an active search for increasing sample size. Using tools such as MALDI TOF–MS for developing sensitive and cost-effective identification strategies has also been a valuable outcome of this study regarding a new health situation being detected regarding one of the infections having a major social impact on lives and economic impact on resources for the Colombian healthcare system.

This work's implications were related to three key aspects. The first aspect concerned methods for characterising patterns of susceptibility and resistance to new antifungal drugs

(i.e. echinocandins), especially for nosocomial clinical isolates. The findings described herein indicated the need for further epidemiological studies involving the latest methodology (CLSI M27-S4); agreement with other tests, such as the EUCAST and E-tests, was established, thereby enabling uniform parameters between hospitals to be compared, as changes in echinocandin susceptibility were observed in the isolates described here and resistance was observed in one of them without mutations in the *FKS1* gene which has been the commonest cause of resistance to date.

The second key aspect concerned correct *Candida albicans* species' classification, especially regarding the need for a better definition for identifying nosocomial atypical species, as this also affects the epidemiological characterisation of infection, as well as revealing phenotypical differences associated with cell wall pattern. Further studies in this field are thus required.

The third key aspect concerned the clinical significance of Candida infection and its relationship with the nutritional and social conditions of patients affected by this opportunistic microorganism.

An overall implication worth highlighting was related to the concept of a nosocomial germ as a microorganism behaving in line with the biological conditions in which it adapts, grows and infects susceptible hosts. This underlines a new R&D field aimed at improving hospitals' therapeutic ability and infection control.

Bibliography

1. Whittington A: **From commensal to pathogen: Candida albicans**. In: *Human Fungal Pathogens.* Edited by Kurzai O. Berlin Heidelberg: Springer-Verlag; 2014: 3-18.

2. Pfaller MA, Diekema DJ: **Epidemiology of invasive mycoses in North America**. *Critical reviews in microbiology* 2010 **36**(1):1-53.

3. Pfaller MA: **Nosocomial candidiasis: emerging species, reservoirs, and modes of transmission**. *Clin Infect Dis* 1996, **22 Suppl 2**:S89-94.

4. Wisplinghoff H, Bischoff T, Tallent SM, Seifert H, Wenzel RP, Edmond MB: **Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study**. *Clin Infect Dis* 2004, **39**(3):309-317.

5. Gudlaugsson O, Gillespie S, Lee K, Vande Berg J, Hu J, Messer S, Herwaldt L, Pfaller M, Diekema D: **Attributable mortality of nosocomial candidemia, revisited**. *Clin Infect Dis* 2003, **37**(9):1172-1177.

6. Pfaller MA, Diekema DJ: **Epidemiology of invasive candidiasis: a persistent public health problem**. *Clinical microbiology reviews* 2007, **20**(1):133-163.

7. DiazGranados CA, Martinez A, Deaza C, Valderrama S: **An outbreak of Candida spp. bloodstream infection in a tertiary care center in Bogota, Colombia**. *Braz J Infect Dis* 2008, **12**(5):390-394.

8. Sullivan DJ, Moran GP, Pinjon E, Al-Mosaid A, Stokes C, Vaughan C, Coleman DC: **Comparison of the epidemiology, drug resistance mechanisms, and virulence of Candida dubliniensis and Candida albicans**. *FEMS yeast research* 2004, **4**(4-5):369-376.

9. Pfaller MA, Diekema DJ, Rinaldi MG, Barnes R, Hu B, Veselov AV, Tiraboschi N, Nagy E, Gibbs DL: **Results from the ARTEMIS DISK Global Antifungal Surveillance Study: a 6.5-year analysis of susceptibilities of Candida and other yeast species to fluconazole and voriconazole by standardized disk diffusion testing**. *Journal of clinical microbiology* 2005, **43**(12):5848-5859.

10. Pfaller MA, Boyken L, Hollis RJ, Kroeger J, Messer SA, Tendolkar S, Diekema DJ: **In vitro susceptibility of invasive isolates of Candida spp. to anidulafungin, caspofungin, and micafungin: six years of global surveillance**. *Journal of clinical microbiology* 2008, **46**(1):150-156.

11. Walker LA, Gow NA, Munro CA: **Fungal echinocandin resistance**. *Fungal Genet Biol* 2010, **47**(2):117-126.

12. Dannaoui E, Desnos-Ollivier M, Garcia-Hermoso D, Grenouillet F, Cassaing S, Baixench MT, Bretagne S, Dromer F, Lortholary O, French Mycoses Study G: **Candida spp. with acquired echinocandin resistance, France, 2004-2010**. *Emerging infectious diseases* 2012, **18**(1):86-90.

13. Pappas PG, Kauffman CA, Andes D, Benjamin DK, Jr., Calandra TF, Edwards JE, Jr., Filler SG, Fisher JF, Kullberg BJ, Ostrosky-Zeichner L *et al*: **Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America**. *Clin Infect Dis* 2009, **48**(5):503-535.

14. Shapiro RS, Robbins N, Cowen LE: **Regulatory circuitry governing fungal development, drug resistance, and disease**. *Microbiol Mol Biol Rev* 2011, **75**(2):213-267.

15. Park S, Kelly R, Kahn JN, Robles J, Hsu MJ, Register E, Li W, Vyas V, Fan H, Abruzzo G *et al*: **Specific substitutions in the echinocandin target Fks1p account for reduced susceptibility of rare laboratory and clinical Candida sp. isolates**. *Antimicrobial agents and chemotherapy* 2005, **49**(8):3264-3273.

16. Sorgo AG, Heilmann CJ, Dekker HL, Bekker M, Brul S, de Koster CG, de Koning LJ, Klis FM: **Effects of fluconazole on the secretome, the wall proteome, and wall integrity of the clinical fungus Candida albicans**. *Eukaryotic cell* 2011, **10**(8):1071-1081.

17. Brandt ME: **Recent taxonomic developments with Candida and other opportunistic yeasts.** *Curr Fungal Infect Rep* 2012, **6**(3):170-177.

18. Hedges SB: **The origin and evolution of model organisms**. *Nature reviews Genetics* 2002, **3**(11):838-849.

19. Criseo G, Scordino F, Romeo O: **Current methods for identifying clinically important cryptic Candida species**. *Journal of microbiological methods* 2015, **111C**:50-56.

20. Ngouana TK, Krasteva D, Drakulovski P, Toghueo RK, Kouanfack C, Ambe A, Reynes J, Delaporte E, Boyom FF, Mallie M *et al*: **Investigation of minor species Candida africana, Candida stellatoidea and Candida dubliniensis in the Candida albicans complex among Yaounde (Cameroon) HIV-infected patients**. *Mycoses* 2015, **58**(1):33-39.

21. Tietz HJ, Hopp M, Schmalreck A, Sterry W, Czaika V: **Candida africana sp. nov., a new human pathogen or a variant of Candida albicans?** *Mycoses* 2001, **44**(11-12):437-445.

22. Berman J, Sudbery PE: **Candida Albicans: a molecular revolution built on lessons from budding yeast**. *Nature reviews Genetics* 2002, **3**(12):918-930.

23. Sudbery PE: **Growth of Candida albicans hyphae**. *Nature reviews* 2011, **9**(10):737-748.

24. Wahab AA, Taj-Aldeen SJ, Kolecka A, ElGindi M, Finkel JS, Boekhout T: **High prevalence of Candida dubliniensis in lower respiratory tract secretions from cystic fibrosis patients may be related to increased adherence properties**. *International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases* 2014, **24**:14-19.

25. Sullivan DJ, Westerneng TJ, Haynes KA, Bennett DE, Coleman DC: **Candida dubliniensis sp. nov.: phenotypic and molecular characterization of a novel species associated with oral candidosis in HIV-infected individuals**. *Microbiology* 1995, **141 (Pt 7)**:1507-1521.

26. Jones T, Federspiel NA, Chibana H, Dungan J, Kalman S, Magee BB, Newport G, Thorstenson YR, Agabian N, Magee PT *et al*: **The diploid genome sequence of Candida albicans**. *Proceedings of the National Academy of Sciences of the United States of America* 2004, **101**(19):7329-7334.

27. Butler G, Rasmussen MD, Lin MF, Santos MA, Sakthikumar S, Munro CA, Rheinbay E, Grabherr M, Forche A, Reedy JL *et al*: **Evolution of pathogenicity and sexual reproduction in eight Candida genomes**. *Nature* 2009, **459**(7247):657-662.

28. De Backer MD, Magee PT, Pla J: **Recent developments in molecular genetics of Candida albicans**. *Annual review of microbiology* 2000, **54**:463-498.

29. Santos MA, Tuite MF: **The CUG codon is decoded in vivo as serine and not leucine in Candida albicans**. *Nucleic acids research* 1995, **23**(9):1481-1486.

30. Arnaud MB, Inglis DO, Skrzypek MS, Binkley J, Shah P, Wymore F, Binkley G, Miyasato SR, Simison M, Sherlock G: **Candida Genome Database. http://www.candidagenome.org/ (Last accessed may 19th 2015)**.

31. Forche A, Alby K, Schaefer D, Johnson AD, Berman J, Bennett RJ: **The parasexual cycle in Candida albicans provides an alternative pathway to meiosis for the formation of recombinant strains**. *PLoS biology* 2008, **6**(5):e110.

32. Ruiz-Herrera J, Elorza MV, Valentin E, Sentandreu R: **Molecular organization of the cell wall of Candida albicans and its relation to pathogenicity**. *FEMS yeast research* 2006, **6**(1):14-29.

33. Brown JA, Catley BJ: **Monitoring polysaccharide synthesis in Candida albicans**. *Carbohydrate Research* 1992, **227**:195-202.

34. Gow NA, van de Veerdonk FL, Brown AJ, Netea MG: **Candida albicans morphogenesis and host defence: discriminating invasion from colonization**. *Nature reviews* 2012, **10**(2):112-122.

35. Chaffin WL: **Candida albicans cell wall proteins**. *Microbiol Mol Biol Rev* 2008, **72**(3):495-544.

36. Plaine A, Walker L, Da Costa G, Mora-Montes HM, McKinnon A, Gow NA, Gaillardin C, Munro CA, Richard ML: **Functional analysis of Candida albicans GPI-anchored proteins: roles in cell wall integrity and caspofungin sensitivity**. *Fungal Genet Biol* 2008, **45**(10):1404-1414.

37. Bruno VM, Kalachikov S, Subaran R, Nobile CJ, Kyratsous C, Mitchell AP: **Control of the C. albicans cell wall damage response by transcriptional regulator Cas5**. *PLoS pathogens* 2006, **2**(3):e21.

38. Liu TT, Lee RE, Barker KS, Lee RE, Wei L, Homayouni R, Rogers PD: **Genome-wide expression profiling of the response to azole, polyene, echinocandin, and pyrimidine antifungal agents in Candida albicans**. *Antimicrobial agents and chemotherapy* 2005, **49**(6):2226-2236.

39. Barchiesi F, Orsetti E, Gesuita R, Skrami E, Manso E, Candidemia Study G: **Epidemiology, clinical characteristics, and outcome of candidemia in a tertiary referral center in Italy from 2010 to 2014**. *Infection* 2015.

40. Diekema D, Arbefeville S, Boyken L, Kroeger J, Pfaller M: **The changing epidemiology of healthcare-associated candidemia over three decades**. *Diagnostic microbiology and infectious disease* 2012, **73**(1):45-48.

41. Nucci M, Queiroz-Telles F, Tobon AM, Restrepo A, Colombo AL: **Epidemiology of opportunistic fungal infections in Latin America**. *Clin Infect Dis* 2010, **51**(5):561-570.

42. Chen PY, Chuang YC, Wang JT, Sheng WH, Yu CJ, Chu CC, Hsueh PR, Chang SC, Chen YC: **Comparison of epidemiology and treatment outcome of patients with candidemia at a teaching hospital in Northern Taiwan, in 2002 and 2010**. *Journal of microbiology, immunology, and infection = Wei mian yu gan ran za zhi* 2014, **47**(2):95-103.

43. Wilson LS, Reyes CM, Stolpman M, Speckman J, Allen K, Beney J: **The direct cost and incidence of systemic fungal infections**. *Value Health* 2002, **5**(1):26-34.

44. Blot SI, Depuydt P, Annemans L, Benoit D, Hoste E, De Waele JJ, Decruyenaere J, Vogelaers D, Colardyn F, Vandewoude KH: **Clinical and economic outcomes in critically ill patients with nosocomial catheter-related bloodstream infections**. *Clin Infect Dis* 2005, **41**(11):1591-1598.

45. Moran C, Grussemeyer CA, Spalding JR, Benjamin DK, Jr., Reed SD: **Comparison of costs, length of stay, and mortality associated with Candida glabrata and Candida albicans bloodstream infections**. *American journal of infection control* 2010, **38**(1):78-80.

46. Calderone RA: **Candida and candidiasis**, 2nd edn. Washington D.C., U.S.A.; 2012.

47. Kadosh D, Johnson AD: **Induction of the Candida albicans filamentous growth program by relief of transcriptional repression: a genome-wide analysis**. *Molecular biology of the cell* 2005, **16**(6):2903-2912.

48. Longo DL, Fauci AS, Kasper DL, Hauser SL, Jameson JL, Loscalzo J: **Harrison's principles of internal medicine**, 18th edn: McGraw-Hill; 2012.

49. Hermsen ED, Zapapas MK, Maiefski M, Rupp ME, Freifeld AG, Kalil AC: **Validation and comparison of clinical prediction rules for invasive candidiasis in intensive care unit patients: a matched case-control study**. *Critical care (London, England)* 2011, **15**(4):R198.

50. Garner JS: **Infection control and applied epidemiology**. St. Louis, MI, U.S.A.: Mosby; 1996.

51. Horan TC, Andrus M, Dudeck MA: **CDC/NHSN surveillance definition of health care-associated infection and criteria for specific types of infections in the acute care setting**. *American journal of infection control* 2008, **36**(5):309-332.

52. Perlroth J, Choi B, Spellberg B: **Nosocomial fungal infections: epidemiology, diagnosis, and treatment**. *Med Mycol* 2007, **45**(4):321-346.

53. Sydnor ER, Perl TM: **Hospital epidemiology and infection control in acute-care settings**. *Clinical microbiology reviews* 2011, **24**(1):141-173.

54. Ostrosky-Zeichner L, Casadevall A, Galgiani JN, Odds FC, Rex JH: **An insight into the antifungal pipeline: selected new molecules and beyond**. *Nat Rev Drug Discov* 2010, **9**(9):719-727.

55. Maubon D, Garnaud C, Calandra T, Sanglard D, Cornet M: **Resistance of Candida spp. to antifungal drugs in the ICU: where are we now?** *Intensive care medicine* 2014, **40**(9):1241-1255.

56. Anderson TM, Clay MC, Cioffi AG, Diaz KA, Hisao GS, Tuttle MD, Nieuwkoop AJ, Comellas G, Maryum N, Wang S *et al*: **Amphotericin forms an extramembranous and fungicidal sterol sponge**. *Nature chemical biology* 2014, **10**(5):400-406.

57. Hope WW, Tabernero L, Denning DW, Anderson MJ: **Molecular mechanisms of primary resistance to flucytosine in Candida albicans**. *Antimicrobial agents and chemotherapy* 2004, **48**(11):4377-4386.

58. Eschenauer G, Depestel DD, Carver PL: **Comparison of echinocandin antifungals**. *Therapeutics and clinical risk management* 2007, **3**(1):71-97.

59. Perlin DS: **Resistance to echinocandin-class antifungal drugs**. *Drug Resist Updat* 2007, **10**(3):121-130.

60. Denning DW: **Echinocandin antifungal drugs**. *Lancet* 2003, **362**(9390):1142-1151.

61. Deresinski SC, Stevens DA: **Caspofungin**. *Clin Infect Dis* 2003, **36**(11):1445-1457.

62. Cornely OA, Bassetti M, Calandra T, Garbino J, Kullberg BJ, Lortholary O, Meersseman W, Akova M, Arendrup MC, Arikan-Akdagli S *et al*: **ESCMID* guideline for the diagnosis and management of Candida diseases 2012: non-neutropenic adult patients**. *Clin Microbiol Infect* 2012, **18 Suppl 7**:19-37.

63. Ullmann AJ, Akova M, Herbrecht R, Viscoli C, Arendrup MC, Arikan-Akdagli S, Bassetti M, Bille J, Calandra T, Castagnola E *et al*: **ESCMID* guideline for the diagnosis and management of Candida diseases 2012: adults with haematological malignancies and after haematopoietic stem cell transplantation (HCT)**. *Clin Microbiol Infect* 2012, **18 Suppl 7**:53-67.

64. Cowen LE: **The evolution of fungal drug resistance: modulating the trajectory from genotype to phenotype**. *Nature reviews* 2008, **6**(3):187-198.

65. Rogers TR: **Antifungal drug resistance: limited data, dramatic impact?** *International journal of antimicrobial agents* 2006, **27 Suppl 1**:7-11.

66. Sanglard D, Coste A, Ferrari S: **Antifungal drug resistance mechanisms in fungal pathogens from the perspective of transcriptional gene regulation**. *FEMS yeast research* 2009, **9**(7):1029-1050.

67. Schuetzer-Muehlbauer M, Willinger B, Krapf G, Enzinger S, Presterl E, Kuchler K: **The Candida albicans Cdr2p ATP-binding cassette (ABC) transporter confers resistance to caspofungin**. *Molecular microbiology* 2003, **48**(1):225-235.

68. Watamoto T, Samaranayake LP, Egusa H, Yatani H, Seneviratne CJ: **Transcriptional regulation of drug-resistance genes in Candida albicans biofilms in response to antifungals**. *Journal of medical microbiology* 2011, **60**(Pt 9):1241-1247.

69. Perlin DS: **Current perspectives on echinocandin class drugs**. *Future microbiology* 2011, **6**(4):441-457.

70. Ramage G, Saville SP, Thomas DP, Lopez-Ribot JL: **Candida biofilms: an update**. *Eukaryotic cell* 2005, **4**(4):633-638.

71. Ramage G, Martinez JP, Lopez-Ribot JL: **Candida biofilms on implanted biomaterials: a clinically significant problem**. *FEMS yeast research* 2006, **6**(7):979-986.

72. Singh SD, Robbins N, Zaas AK, Schell WA, Perfect JR, Cowen LE: **Hsp90 governs echinocandin resistance in the pathogenic yeast Candida albicans via calcineurin**. *PLoS pathogens* 2009, **5**(7):e1000532.

73. Ramage G, Rajendran R, Sherry L, Williams C: **Fungal biofilm resistance**. *International journal of microbiology* 2012, **2012**:528521.

74. Laverdiere M, Lalonde RG, Baril JG, Sheppard DC, Park S, Perlin DS: **Progressive loss of echinocandin activity following prolonged use for treatment of Candida albicans oesophagitis**. *The Journal of antimicrobial chemotherapy* 2006, **57**(4):705-708.

75. Garcia-Effron G, Park S, Perlin DS: **Correlating echinocandin MIC and kinetic inhibition of fks1 mutant glucan synthases for Candida albicans: implications for interpretive breakpoints**. *Antimicrobial agents and chemotherapy* 2009, **53**(1):112-122.

76. Balashov SV, Park S, Perlin DS: **Assessing resistance to the echinocandin antifungal drug caspofungin in Candida albicans by profiling mutations in FKS1**. *Antimicrobial agents and chemotherapy* 2006, **50**(6):2058-2063.

77. Miller CD, Lomaestro BW, Park S, Perlin DS: **Progressive esophagitis caused by Candida albicans with reduced susceptibility to caspofungin**. *Pharmacotherapy* 2006, **26**(6):877-880.

78. Katiyar S, Pfaller M, Edlind T: **Candida albicans and Candida glabrata clinical isolates exhibiting reduced echinocandin susceptibility**. *Antimicrobial agents and chemotherapy* 2006, **50**(8):2892-2894.

79. Baixench MT, Aoun N, Desnos-Ollivier M, Garcia-Hermoso D, Bretagne S, Ramires S, Piketty C, Dannaoui E: **Acquired resistance to echinocandins in Candida albicans: case report and review**. *The Journal of antimicrobial chemotherapy* 2007, **59**(6):1076-1083.

80. Pfaller MA, Diekema DJ, Andes D, Arendrup MC, Brown SD, Lockhart SR, Motyl M, Perlin DS: **Clinical breakpoints for the echinocandins and Candida revisited: integration of molecular, clinical, and microbiological data to arrive at species-specific interpretive criteria**. *Drug Resist Updat* 2011, **14**(3):164-176.

81. Wiederhold NP, Grabinski JL, Garcia-Effron G, Perlin DS, Lee SA: **Pyrosequencing to detect mutations in FKS1 that confer reduced echinocandin susceptibility in Candida albicans**. *Antimicrobial agents and chemotherapy* 2008, **52**(11):4145-4148.

82. Poulain D, Jouault T: **Candida albicans cell wall glycans, host receptors and responses: elements for a decisive crosstalk**. *Current opinion in microbiology* 2004, **7**(4):342-349.

83. Nather K, Munro CA: **Generating cell surface diversity in Candida albicans and other fungal pathogens**. *FEMS microbiology letters* 2008, **285**(2):137-145.

84. Cowen LE, Steinbach WJ: **Stress, drugs, and evolution: the role of cellular signaling in fungal drug resistance**. *Eukaryotic cell* 2008, **7**(5):747-764.

85. Angiolella L, Stringaro AR, De Bernardis F, Posteraro B, Bonito M, Toccacieli L, Torosantucci A, Colone M, Sanguinetti M, Cassone A *et al*: **Increase of virulence and its phenotypic traits in drug-resistant strains of Candida albicans**. *Antimicrobial agents and chemotherapy* 2008, **52**(3):927-936.

86. Wiederhold NP, Kontoyiannis DP, Prince RA, Lewis RE: **Attenuation of the activity of caspofungin at high concentrations against candida albicans: possible role of cell wall integrity and calcineurin pathways**. *Antimicrobial agents and chemotherapy* 2005, **49**(12):5146-5148.

87. Rauceo JM, Blankenship JR, Fanning S, Hamaker JJ, Deneault JS, Smith FJ, Nantel A, Mitchell AP: **Regulation of the Candida albicans cell wall damage response by transcription factor Sko1 and PAS kinase Psk1**. *Molecular biology of the cell* 2008, **19**(7):2741-2751.

88. Onyewu C, Wormley FL, Jr., Perfect JR, Heitman J: **The calcineurin target, Crz1, functions in azole tolerance but is not required for virulence of Candida albicans**. *Infection and immunity* 2004, **72**(12):7330-7333.

89. Perlin DS: **Echinocandin resistance, susceptibility testing and prophylaxis: implications for patient management**. *Drugs* 2014, **74**(14):1573-1585.

90. Shields RK, Nguyen MH, Du C, Press E, Cheng S, Clancy CJ: **Paradoxical effect of caspofungin against Candida bloodstream isolates is mediated by multiple pathways but eliminated in human serum**. *Antimicrobial agents and chemotherapy* 2011, **55**(6):2641-2647.

91. Stevens DA, Ichinomiya M, Koshi Y, Horiuchi H: **Escape of Candida from caspofungin inhibition at concentrations above the MIC (paradoxical effect) accomplished by increased cell wall chitin; evidence for beta-1,6-glucan synthesis inhibition by caspofungin**. *Antimicrobial agents and chemotherapy* 2006, **50**(9):3160-3161.

92. Gauwerky K, Borelli C, Korting HC: **Targeting virulence: a new paradigm for antifungals**. *Drug discovery today* 2009, **14**(3-4):214-222.

93. Wilson D, Thewes S, Zakikhany K, Fradin C, Albrecht A, Almeida R, Brunke S, Grosse K, Martin R, Mayer F *et al*: **Identifying infection-associated genes of Candida albicans in the postgenomic era**. *FEMS yeast research* 2009, **9**(5):688-700.

94. Nailis H, Kucharikova S, Ricicova M, Van Dijck P, Deforce D, Nelis H, Coenye T: **Real-time PCR expression profiling of genes encoding potential virulence factors in Candida albicans biofilms: identification of model-dependent and -independent gene expression**. *BMC microbiology* 2010, **10**:114.

95. Felk A, Kretschmar M, Albrecht A, Schaller M, Beinhauer S, Nichterlein T, Sanglard D, Korting HC, Schafer W, Hube B: **Candida albicans hyphal formation and the expression of the Efg1-regulated proteinases Sap4 to Sap6 are required for the invasion of parenchymal organs**. *Infection and immunity* 2002, **70**(7):3689-3700.

96. Wayne PA: **CLSI. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast; CLSI document M27-A3**; 2008.

97. Rodriguez-Tudela JL: **EUCAST definitive document EDef 7.1: method for the determination of broth dilution MICs of antifungal agents for fermentative yeasts**. *Clin Microbiol Infect* 2008, **14**(4):398-405.

98. Espinel-Ingroff A, Arendrup MC, Pfaller MA, Bonfietti LX, Bustamante B, Canton E, Chryssanthou E, Cuenca-Estrella M, Dannaoui E, Fothergill A *et al*: **Interlaboratory variability of Caspofungin MICs for Candida spp. Using CLSI and EUCAST methods: should the clinical laboratory be testing this agent?** *Antimicrobial agents and chemotherapy* 2013, **57**(12):5836-5842.

99. Wayne PA: **CLSI. Reference Method for Broth Dilution Antifungal Suscetibility Testing of Yeasts; Fourth Informational Supplement. CLSI Document M27-S4**; 2012.

100. Pfaller MA, Diekema DJ, Ostrosky-Zeichner L, Rex JH, Alexander BD, Andes D, Brown SD, Chaturvedi V, Ghannoum MA, Knapp CC *et al*: **Correlation of MIC with outcome for Candida species tested against caspofungin, anidulafungin, and micafungin: analysis and proposal for interpretive MIC breakpoints**. *Journal of clinical microbiology* 2008, **46**(8):2620-2629.

101. Espinel-Ingroff A: **In vitro antifungal activities of anidulafungin and micafungin, licensed agents and the investigational triazole posaconazole as determined by NCCLS methods for 12,052 fungal isolates: review of the literature**. *Rev Iberoam Micol* 2003, **20**(4):121-136.

102. Ostrosky-Zeichner L, Rex JH, Pappas PG, Hamill RJ, Larsen RA, Horowitz HW, Powderly WG, Hyslop N, Kauffman CA, Cleary J *et al*: **Antifungal susceptibility survey of 2,000 bloodstream Candida isolates in the United States**. *Antimicrobial agents and chemotherapy* 2003, **47**(10):3149-3154.

103. Espinel-Ingroff A, Canton E, Peman J, Martin-Mazuelo E: **Comparison of anidulafungin MICs determined by the clinical and laboratory standards institute broth microdilution method (M27-A3 document) and Etest for Candida species isolates**. *Antimicrobial agents and chemotherapy* 2010, **54**(3):1347-1350.

104. Pfaller MA, Castanheira M, Diekema DJ, Messer SA, Moet GJ, Jones RN: **Comparison of European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Etest methods with the CLSI broth microdilution method for echinocandin susceptibility testing of Candida species**. *Journal of clinical microbiology* 2010, **48**(5):1592-1599.

105. Pfaller MA, Boyken L, Hollis RJ, Kroeger J, Messer SA, Tendolkar S, Jones RN, Turnidge J, Diekema DJ: **Wild-type MIC distributions and epidemiological cutoff values for the echinocandins and Candida spp**. *Journal of clinical microbiology* 2010, **48**(1):52-56.

106. Arendrup MC, Cuenca-Estrella M, Lass-Florl C, Hope WW: **Breakpoints for antifungal agents: an update from EUCAST focussing on echinocandins against Candida spp. and triazoles against Aspergillus spp**. *Drug Resist Updat* 2013, **16**(6):81-95.

107. Pfaller MA, Boyken L, Hollis RJ, Messer SA, Tendolkar S, Diekema DJ: **Global surveillance of in vitro activity of micafungin against Candida: a comparison with caspofungin by CLSI-recommended methods**. *Journal of clinical microbiology* 2006, **44**(10):3533-3538.

108. Arendrup MC, Garcia-Effron G, Lass-Florl C, Lopez AG, Rodriguez-Tudela JL, Cuenca-Estrella M, Perlin DS: **Echinocandin susceptibility testing of Candida species: comparison of EUCAST EDef 7.1, CLSI M27-A3, Etest, disk diffusion, and agar dilution methods with RPMI and isosensitest media**. *Antimicrobial agents and chemotherapy* 2010, **54**(1):426-439.

109. Ministerio de Protección Social: **Resolución 8430**. In. Bogotá, Colombia; 1993.

110. WMA Declaration of Helsinki: **Ethical Principles for Human Research Involving Human Subjects**. In. Seoul, Korea; 2008.

111. Garner J.S: **Infection Control and Applied Epidemiology**. St. Louis: Mosby; 1996.

112. Pfaller MA, Bale M, Buschelman B, Lancaster M, Espinel-Ingroff A, Rex JH, Rinaldi MG: **Selection of candidate quality control isolates and tentative quality control ranges for in vitro susceptibility testing of yeast isolates by National Committee for Clinical Laboratory Standards proposed standard methods**. *Journal of clinical microbiology* 1994, **32**(7):1650-1653.

113. Koszul R, Malpertuy A, Frangeul L, Bouchier C, Wincker P, Thierry A, Duthoy S, Ferris S, Hennequin C, Dujon B: **The complete mitochondrial genome sequence of the pathogenic yeast Candida (Torulopsis) glabrata**. *FEBS letters* 2003, **534**(1-3):39-48.

114. Ramage G, Vandewalle K, Wickes BL, Lopez-Ribot JL: **Characteristics of biofilm formation by Candida albicans**. *Rev Iberoam Micol* 2001, **18**(4):163-170.

115. Maidan MM, De Rop L, Relloso M, Diez-Orejas R, Thevelein JM, Van Dijck P: **Combined inactivation of the Candida albicans GPR1 and TPS2 genes results in avirulence in a mouse model for systemic infection**. *Infection and immunity* 2008, **76**(4):1686-1694.

116. Wilson D, Fiori A, Brucker KD, Dijck PV, Stateva L: **Candida albicans Pde1p and Gpa2p comprise a regulatory module mediating agonist-induced cAMP signalling and environmental adaptation**. *Fungal Genet Biol* 2010, **47**(9):742-752.

117. Cuetara MS, Alhambra A, Del Palacio A: **[Traditional microbiological diagnosis for invasive candidiasis in critical non-neutropenic patients]**. *Rev Iberoam Micol* 2006, **23**(1):4-7.

118. Livak KJ, Schmittgen TD: **Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method**. *Methods* 2001, **25**(4):402-408.

119. Walker LA, Gow NA, Munro CA: **Elevated chitin content reduces the susceptibility of Candida species to caspofungin**. *Antimicrobial agents and chemotherapy* 2013, **57**(1):146-154.

120. Cendejas-Bueno E, Kolecka A, Alastruey-Izquierdo A, Theelen B, Groenewald M, Kostrzewa M, Cuenca-Estrella M, Gomez-Lopez A, Boekhout T: **Reclassification of the Candida haemulonii complex as Candida haemulonii (C. haemulonii group I), C. duobushaemulonii sp. nov. (C. haemulonii group II), and C. haemulonii var. vulnera var. nov.: three multiresistant human pathogenic yeasts**. *Journal of clinical microbiology* 2012, **50**(11):3641-3651.

121. Qian J, Cutler JE, Cole RB, Cai Y: **MALDI-TOF mass signatures for differentiation of yeast species, strain grouping and monitoring of morphogenesis markers**. *Analytical and bioanalytical chemistry* 2008, **392**(3):439-449.

122. Suzuki R, Shimodaira H: **Pvclust: an R package for assessing the uncertainty in hierarchical clustering**. *Bioinformatics* 2006, **22**(12):1540-1542.

123. Alberti-Segui C, Morales AJ, Xing H, Kessler MM, Willins DA, Weinstock KG, Cottarel G, Fechtel K, Rogers B: **Identification of potential cell-surface proteins in Candida albicans and investigation of the role of a putative cell-surface glycosidase in adhesion and virulence**. *Yeast (Chichester, England)* 2004, **21**(4):285-302.

124. White TJ BT, Lee S, Taylor J.: **Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics**. In: *PCR Protocols: A Guide Methods and Applications*. Edited by Innis A GD, Snisky JJ, White TJ. San Diego, CA: Academic Press; 1990: 315-322.

125. Romeo O, Criseo G: **First molecular method for discriminating between Candida africana, Candida albicans, and Candida dubliniensis by using hwp1 gene**. *Diagnostic microbiology and infectious disease* 2008, **62**(2):230-233.

126. Edwards JE, Jr., Bodey GP, Bowden RA, Buchner T, de Pauw BE, Filler SG, Ghannoum MA, Glauser M, Herbrecht R, Kauffman CA *et al*: **International**

Conference for the Development of a Consensus on the Management and Prevention of Severe Candidal Infections. *Clin Infect Dis* 1997, **25**(1):43-59.

127. Eggimann P, Ostrosky-Zeichner L: **Early antifungal intervention strategies in ICU patients**. *Current opinion in critical care* 2010, **16**(5):465-469.

128. Asmundsdottir LR, Erlendsdottir H, Haraldsson G, Guo H, Xu J, Gottfredsson M: **Molecular epidemiology of candidemia: evidence of clusters of smoldering nosocomial infections**. *Clin Infect Dis* 2008, **47**(2):e17-24.

129. Drakulovski P, Dunyach C, Bertout S, Reynes J, Mallie M: **A Candida albicans strain with high MIC for caspofungin and no FKS1 mutations exhibits a high chitin content and mutations in two chitinase genes**. *Med Mycol* 2011, **49**(5):467-474.

130. Gomez J, Garcia-Vazquez E, Hernandez A, Espinosa C, Ruiz J: **[Nosocomial candidemia: new challenges of an emergent problem]**. *Rev Esp Quimioter* 2010, **23**(4):158-168.

131. Morrell M, Fraser VJ, Kollef MH: **Delaying the empiric treatment of candida bloodstream infection until positive blood culture results are obtained: a potential risk factor for hospital mortality**. *Antimicrobial agents and chemotherapy* 2005, **49**(9):3640-3645.

132. Baillie GS, Douglas LJ: **Effect of growth rate on resistance of Candida albicans biofilms to antifungal agents**. *Antimicrobial agents and chemotherapy* 1998, **42**(8):1900-1905.

133. Ernst JF, Pla J: **Signaling the glycoshield: maintenance of the Candida albicans cell wall**. *International journal of medical microbiology : IJMM* 2011, **301**(5):378-383.

134. Brauer MJ, Huttenhower C, Airoldi EM, Rosenstein R, Matese JC, Gresham D, Boer VM, Troyanskaya OG, Botstein D: **Coordination of growth rate, cell cycle, stress response, and metabolic activity in yeast**. *Molecular biology of the cell* 2008, **19**(1):352-367.

135. Kucharikova S, Tournu H, Lagrou K, Van Dijck P, Bujdakova H: **Detailed comparison of Candida albicans and Candida glabrata biofilms under different conditions and their susceptibility to caspofungin and anidulafungin**. *Journal of medical microbiology* 2011, **60**(Pt 9):1261-1269.

136. Staab JF, Bradway SD, Fidel PL, Sundstrom P: **Adhesive and mammalian transglutaminase substrate properties of Candida albicans Hwp1**. *Science* 1999, **283**(5407):1535-1538.

137. Cowen LE, Anderson JB, Kohn LM: **Evolution of drug resistance in Candida albicans**. *Annual review of microbiology* 2002, **56**:139-165.

138. Maidan MM, Thevelein JM, Van Dijck P: **Carbon source induced yeast-to-hypha transition in Candida albicans is dependent on the presence of amino acids and on the G-protein-coupled receptor Gpr1**. *Biochemical Society transactions* 2005, **33**(Pt 1):291-293.

139. Homann OR, Dea J, Noble SM, Johnson AD: **A phenotypic profile of the Candida albicans regulatory network**. *PLoS genetics* 2009, **5**(12):e1000783.

140. Han TL, Cannon RD, Villas-Boas SG: **The metabolic basis of Candida albicans morphogenesis and quorum sensing**. *Fungal Genet Biol* 2011, **48**(8):747-763.

141. Liu H, Kohler J, Fink GR: **Suppression of hyphal formation in Candida albicans by mutation of a STE12 homolog**. *Science* 1994, **266**(5191):1723-1726.

142. Toenjes KA, Munsee SM, Ibrahim AS, Jeffrey R, Edwards JE, Jr., Johnson DI: **Small-molecule inhibitors of the budded-to-hyphal-form transition in the pathogenic yeast Candida albicans**. *Antimicrobial agents and chemotherapy* 2005, **49**(3):963-972.

143. Peeters T, Versele M, Thevelein JM: **Directly from Galpha to protein kinase A: the kelch repeat protein bypass of adenylate cyclase**. *Trends in biochemical sciences* 2007, **32**(12):547-554.

144. Kadosh D, Lopez-Ribot JL: **Candida albicans: adapting to succeed**. *Cell host & microbe* 2013, **14**(5):483-485.

145. Braun BR, Kadosh D, Johnson AD: **NRG1, a repressor of filamentous growth in C.albicans, is down-regulated during filament induction**. *The EMBO journal* 2001, **20**(17):4753-4761.

146. Nobile CJ, Fox EP, Nett JE, Sorrells TR, Mitrovich QM, Hernday AD, Tuch BB, Andes DR, Johnson AD: **A recently evolved transcriptional network controls biofilm development in Candida albicans**. *Cell* 2012, **148**(1-2):126-138.

147. Martel CM, Parker JE, Bader O, Weig M, Gross U, Warrilow AG, Kelly DE, Kelly SL: **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**. *Antimicrobial agents and chemotherapy* 2010, **54**(9):3578-3583.

148. Markovich S, Yekutiel A, Shalit I, Shadkchan Y, Osherov N: **Genomic approach to identification of mutations affecting caspofungin susceptibility in Saccharomyces cerevisiae**. *Antimicrobial agents and chemotherapy* 2004, **48**(10):3871-3876.

149. Barker KS, Crisp S, Wiederhold N, Lewis RE, Bareither B, Eckstein J, Barbuch R, Bard M, Rogers PD: **Genome-wide expression profiling reveals genes associated with amphotericin B and fluconazole resistance in experimentally induced**

antifungal resistant isolates of Candida albicans. *The Journal of antimicrobial chemotherapy* 2004, **54**(2):376-385.

150. Zhang N, Cannon RD, Holland BR, Patchett ML, Schmid J: **Impact of genetic background on allele selection in a highly mutable Candida albicans gene, PNG2**. *PloS one* 2010, **5**(3):e9614.

151. Munro CA, Selvaggini S, de Bruijn I, Walker L, Lenardon MD, Gerssen B, Milne S, Brown AJ, Gow NA: **The PKC, HOG and Ca2+ signalling pathways co-ordinately regulate chitin synthesis in Candida albicans**. *Molecular microbiology* 2007, **63**(5):1399-1413.

152. Walker LA, Munro CA, de Bruijn I, Lenardon MD, McKinnon A, Gow NA: **Stimulation of chitin synthesis rescues Candida albicans from echinocandins**. *PLoS pathogens* 2008, **4**(4):e1000040.

153. Nishiyama Y, Uchida K, Yamaguchi H: **Morphological changes of Candida albicans induced by micafungin (FK463), a water-soluble echinocandin-like lipopeptide**. *Journal of electron microscopy* 2002, **51**(4):247-255.

154. Bader O, Weig M, Taverne-Ghadwal L, Lugert R, Gross U, Kuhns M: **Improved clinical laboratory identification of human pathogenic yeasts by matrix-assisted laser desorption ionization time-of-flight mass spectrometry**. *Clin Microbiol Infect* 2011, **17**(9):1359-1365.

155. Vlek A, Kolecka A, Khayhan K, Theelen B, Groenewald M, Boel E, Boekhout T: **Interlaboratory comparison of sample preparation methods, database expansions, and cutoff values for identification of yeasts by matrix-assisted laser desorption ionization-time of flight mass spectrometry using a yeast test panel**. *Journal of clinical microbiology* 2014, **52**(8):3023-3029.

156. Tan KE, Ellis BC, Lee R, Stamper PD, Zhang SX, Carroll KC: **Prospective evaluation of a matrix-assisted laser desorption ionization-time of flight mass spectrometry system in a hospital clinical microbiology laboratory for identification of bacteria and yeasts: a bench-by-bench study for assessing the impact on time to identification and cost-effectiveness**. *Journal of clinical microbiology* 2012, **50**(10):3301-3308.

157. Alonso-Vargas R, Elorduy L, Eraso E, Cano FJ, Guarro J, Ponton J, Quindos G: **Isolation of Candida africana, probable atypical strains of Candida albicans, from a patient with vaginitis**. *Med Mycol* 2008, **46**(2):167-170.

158. Dieng Y, Sow D, Ndiaye M, Guichet E, Faye B, Tine R, Lo A, Sylla K, Ndiaye M, Abiola A *et al*: **[Identification of three Candida africana strains in Senegal]**. *Journal de mycologie medicale* 2012, **22**(4):335-340.

159. Nnadi NE, Ayanbimpe GM, Scordino F, Okolo MO, Enweani IB, Criseo G, Romeo O: **Isolation and molecular characterization of Candida africana from Jos, Nigeria**. *Med Mycol* 2012, **50**(7):765-767.

160. Romeo O, Criseo G: **Morphological, biochemical and molecular characterisation of the first Italian Candida africana isolate**. *Mycoses* 2009, **52**(5):454-457.

161. Odds FC, Bougnoux ME, Shaw DJ, Bain JM, Davidson AD, Diogo D, Jacobsen MD, Lecomte M, Li SY, Tavanti A *et al*: **Molecular phylogenetics of Candida albicans**. *Eukaryotic cell* 2007, **6**(6):1041-1052.

162. Romeo O TH, Criseo G.: **Candida africana: It is a fungal pathogen?** *Curr Fungal Infect Rep* 2013, **7**:192-197.

163. Pulcrano G, Iula DV, Vollaro A, Tucci A, Cerullo M, Esposito M, Rossano F, Catania MR: **Rapid and reliable MALDI-TOF mass spectrometry identification of Candida non-albicans isolates from bloodstream infections**. *Journal of microbiological methods* 2013, **94**(3):262-266.

164. De Carolis E, Vella A, Vaccaro L, Torelli R, Posteraro P, Ricciardi W, Sanguinetti M, Posteraro B: **Development and validation of an in-house database for matrix-assisted laser desorption ionization-time of flight mass spectrometry-based yeast identification using a fast protein extraction procedure**. *Journal of clinical microbiology* 2014, **52**(5):1453-1458.

165. Pedreno Y, Maicas S, Arguelles JC, Sentandreu R, Valentin E: **The ATC1 gene encodes a cell wall-linked acid trehalase required for growth on trehalose in Candida albicans**. *The Journal of biological chemistry* 2004, **279**(39):40852-40860.

166. Ram SP, Romana LK, Shepherd MG, Sullivan PA: **Exo-(1----3)-beta-glucanase, autolysin and trehalase activities during yeast growth and germ-tube formation in Candida albicans**. *Journal of general microbiology* 1984, **130**(5):1227-1236.

167. Peterson SW, Kurtzman CP: **Ribosomal RNA sequence divergence among sibling species of yeasts**. *Syst Appl Microbiol* 1991, **14**:124-129.

168. Borman AM, Szekely A, Linton CJ, Palmer MD, Brown P, Johnson EM: **Epidemiology, antifungal susceptibility, and pathogenicity of Candida africana isolates from the United Kingdom**. *Journal of clinical microbiology* 2013, **51**(3):967-972.

169. Shan Y, Fan S, Liu X, Li J: **Prevalence of Candida albicans-closely related yeasts, Candida africana and Candida dubliniensis, in vulvovaginal candidiasis**. *Med Mycol* 2014, **52**(6):636-640.

170. Pukkila-Worley R, Peleg AY, Tampakakis E, Mylonakis E: **Candida albicans hyphal formation and virulence assessed using a Caenorhabditis elegans infection model**. *Eukaryotic cell* 2009, **8**(11):1750-1758.

171. Beck-Sague C, Jarvis WR: **Secular trends in the epidemiology of nosocomial fungal infections in the United States, 1980-1990. National Nosocomial Infections Surveillance System**. *The Journal of infectious diseases* 1993, **167**(5):1247-1251.

172. Gibbons JD: **Nonparametric statistical inference**. New York: McGraw Hill; 1971.

173. Castellan NJ, Siegel S: **Nonparametric statistics for the behavioral science**, 2nd edn: McGraw - Hill Publishing Co; 1988.

174. Yang SP, Chen YY, Hsu HS, Wang FD, Chen LY, Fung CP: **A risk factor analysis of healthcare-associated fungal infections in an intensive care unit: a retrospective cohort study**. *BMC infectious diseases* 2013, **13**:10.

175. Chandra RK: **Impact of nutritional status and nutrient supplements on immune responses and incidence of infection in older individuals**. *Ageing research reviews* 2004, **3**(1):91-104.

176. Ostrosky-Zeichner L, Pappas PG, Shoham S, Reboli A, Barron MA, Sims C, Wood C, Sobel JD: **Improvement of a clinical prediction rule for clinical trials on prophylaxis for invasive candidiasis in the intensive care unit**. *Mycoses* 2009, **54**(1):46-51.

177. Bastert J, Schaller M, Korting HC, Evans EG: **Current and future approaches to antimycotic treatment in the era of resistant fungi and immunocompromised hosts**. *International journal of antimicrobial agents* 2001, **17**(2):81-91.

178. Diekema DJ, Pfaller MA: **Nosocomial candidemia: an ounce of prevention is better than a pound of cure**. *Infect Control Hosp Epidemiol* 2004, **25**(8):624-626.

179. Sobel JD, Rex JH: **Invasive candidiasis: turning risk into a practical prevention policy?** *Clin Infect Dis* 2001, **33**(2):187-190.

180. Rodriguez-Leguizamon G, Fiori A, Lagrou K, Gaona MA, Ibanez M, Patarroyo MA, Van Dijck P, Gomez-Lopez A: **New echinocandin susceptibility patterns for nosocomial Candida albicans in Bogota, Colombia, in ten tertiary care centres: an observational study**. *BMC infectious diseases* 2015, **15**:108.

181. Ben-Ami R, Garcia-Effron G, Lewis RE, Gamarra S, Leventakos K, Perlin DS, Kontoyiannis DP: **Fitness and virulence costs of Candida albicans FKS1 hot spot mutations associated with echinocandin resistance**. *The Journal of infectious diseases* 2011, **204**(4):626-635.

182. Slater JL, Howard SJ, Sharp A, Goodwin J, Gregson LM, Alastruey-Izquierdo A, Arendrup MC, Warn PA, Perlin DS, Hope WW: **Disseminated Candidiasis caused by Candida albicans with amino acid substitutions in Fks1 at position Ser645 cannot be successfully treated with micafungin**. *Antimicrobial agents and chemotherapy* 2011, **55**(7):3075-3083.

183. Romeo O, Criseo G: **Candida africana and its closest relatives**. *Mycoses* 2011, **54**(6):475-486.

184. Gil-Alonso S, Jauregizar N, Canton E, Eraso E, Quindos G: **Comparison of the in vitro activity of echinocandins against Candida albicans, Candida dubliniensis, and Candida africana by time-kill curves**. *Diagnostic microbiology and infectious disease* 2015, **82**(1):57-61.