MECHANISMS OF ANTIFUNGAL RESISTANCE IN PATHOGENIC YEASTS:

EVALUATION OF THE *IN VITRO* AND *IN VIVO* EXPRESSION

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List of Publications

Ao Abrigo do Art. 8° do Decreto –Lei ° 388/ 70 fazem parte integrante desta dissertação os seguintes trabalhos já publicados, ou em vias de publicação:

Manuscripts

I. **Costa-de-Oliveira S**, Sampaio-Marques B, Barbosa M, Ricardo E, Pina-Vaz C, Ludovico P, Rodrigues AG. An Alternative Respiratory Pathway on Candida krusei: Implications on Susceptibility Profile and oxidative stress. *FEMS Yeast Res*. 2012 Jan 23. doi: 10.1111/j.1567-1364.2012.00789.

II. **Costa-de-Oliveira S**, Miranda I, Silva R, Silva AP, Rocha R, Amorim A, Rodrigues AG, Pina-Vaz C. *FKS2* mutations associated with decreased echinocandin susceptibility of *Candida glabrata* following anidulafungin therapy. *Antimicrobial Agents and Chemotherapy* 2011; 55:1312-1314.

III. **Costa-de-Oliveira S**, Sousa I, Correia A, Sampaio P, Pais C, Rodrigues AG, Pina-Vaz C. Genetic relatedness and antifungal susceptibility profile of *Candida albicans* isolates from fungaemia patients. *Medical Mycology* 2011; 3:248-52.

IV. **Costa de Oliveira S**, Araujo R, Silva-Dias A, Pina Vaz C, Rodrigues AG. Propofol lipidic infusion promotes resistance to antifungals by reducing drug input into the fungal cell. *BMC Microbiology* 2008; 17:8-9.

V. Ricardo E, Silva AP, Gonçalves T, **Costa-de-Oliveira S**, Granato C, Martins J, Rodrigues AG, Pina-Vaz C. *Candida krusei* reservoir in a neutropaenia unit: molecular evidence of a foe? *Clinical Microbiology and Infection* 2011; 17:259-263.

VI. Sampaio P, Santos M, Correia A, Amaral FE, Chavéz-Galarza J, **Costa-de-Oliveira S**, Castro AG, Pedrosa J, Pais C. Virulence attenuation of *Candida albicans* genetic variants isolated from a patient with a recurrent bloodstream infection. *PLoS One*. 13;5(4):e10155, 2010.

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VIII. Ricardo E, **Costa-de-Oliveira S**, Dias AS, Guerra J, Rodrigues AG, Pina-Vaz C. Ibuprofen reverts antifungal resistance on *Candida albicans* showing overexpression of CDR genes. *FEMS Yeast Research* 2009; 9:618-25.

IX. Pinto e Silva AT, **Costa-de-Oliveira S**, Silva-Dias A, Pina-Vaz C, Rodrigues AG. "Dynamics of *in vitro* acquisition of resistance by *Candida parapsilosis* to different azoles. *FEMS Yeast Research* 2009; 9:626-33.

X. Araujo R, **Costa-de-Oliveira S**, Coutinho I, Rodrigues AG, Pina-Vaz C. Evaluating the resistance to posaconazole by E-test and CLSI broth microdilution methodologies of *Candida* spp. and pathogenic moulds. *European Journal of Clinical Microbiology and Infectious Diseases* 2009; 28:1137-40.

XI. Araujo R, Carneiro A, **Costa de Oliveira S**, Pina Vaz C, Rodrigues AG, Guimarães, JE. Fungal infections after haematology unit renovation: evidence of clinical, environmental and economical impact. European *Journal of Haematology* 2008; 80: 436-43.

XII. **Sofia Costa-de-Oliveira**, Isabel Marcos Miranda, Ana Silva-Dias, Cidália Pina-Vaz, Helder Pinheiro, Daniel Moura, Dominique Sanglard, Acácio G. Rodrigues. Adrenaline stimulates efflux pumps activity, growth and mitochondrial respiration in *Candida albicans.* (submitted)

XIII. **Sofia Costa-de-Oliveira**, Isabel Marcos Miranda, Elisabete Ricardo, Ana Silva-Dias, Cidália Pina-Vaz, Acácio G. Rodrigues. *In vivo* synergistic effect between ibuprofen and fluconazole in *Candida albicans*. (submitted).

XIV. **Sofia Costa-de-Oliveira**, Ana P. Silva, Isabel M. Miranda, Alexandre Salvador, Maria M Azevedo, Carol A. Munro, Acácio G. Rodrigues, Cidália Pina-Vaz. Determination of chitin content in fungal cell wall: an alternative flow cytometric method. (submitted)

Abstracts

I. **Costa-de-Oliveira S**, Miranda IM, Ricardo E, Silva-Dias A, Rodrigues AG, Pina-Vaz C. Effective reversion of fluconazole resistance by ibuprofen in an animal model. *Clin Microb Infect* 2012. (in press)

II. **Costa de Oliveira S**, IM Miranda, A Silva-Dias, C. Pina Vaz, D. Moura, AG Rodrigues. Adrenaline enhances yeast cell growth and ATP production through a common target to mammalian cells. *Mycoses* 2011; 54:166.

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IV. Silva A., **Costa de Oliveira S.**, Miranda I, Pina Vaz C, Rodrigues AG. Fungaemia by *Candida parapsilosis*: *in vivo* induction of azole resistance due to prolonged therapeutic exposure. *Clin Microbiol Infect*. 2010; 16: S216.

List of abbreviations

ABC	Adenosine triphosphate Binding Cassette
ADR	Adrenaline
AIDS	Acquired Immune Deficiency Syndrome
AMB lipo	Amphtericin B Lipid Complex
AND	Anidulafungin
AOX	Alternative Oxidase
ARE	Azole-Responsive enhancer
ARP	Alternative Respiratory Pathway
CaR	*Candida albicans* azole resistant induced strain
CaS	*Candida albicans* azole susceptible
CDC	Centre of Disease Control and Prevention
CDR	*Candida* Drug Resistance
CFS	Caspofungin
CFU	Colony Forming Unit
CFW	Calcofluor White
Chr5	Chromosome 5
CLSI	Clinical Laboratory Standards Institute
CyA	Clyclosporine A
Cyp	Cyclophilin
DHR 123	Dihydrorhodamine 123
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide Triphosphate
DST	Diploid Sequence Type
EC50	Half Maximal Effective Concentration
ECV	Epidemiological Cutoff Value
ED50	Half Maximal effective Dose
FC	Flow Cytometry
FIC	Fractional Inhibitory Concentration
FIX	Fractional Inhibitory Index
FK 506	Tacrolimus
FLC	Fluconazole
GPCR	G Protein-Coupled Receptor
GPI	Glycosylphosphatidylinositol
GTP	Guanosine Triphosphate
HOG	High Osmolarity Glycerol Response
HS	Hot Spot
HSP	Heat Shock Protein

Ibu	Ibuprofen
ICU	Intensive Care Unit
ITC	Itraconazole
LOH	Loss of Heterozygosity
MAP	Mitogen-Activated Protein
MCA	Micafungin
MDR	Multi Drug Resistance
MF	Major Facilitator
MFS	Major Facilitator Superfamily
MIC	Minimal inhibitory Concentration
MLC	Minimal Lethal Concentration
MLP	Microsatellite Length Polymorphism
MLST	Multilocus Sequence Typing
MTL	Mating-Type Locus
NCCLS	National Clinical Collaborative Laboratory Standards
NS	Non-Susceptible
NSAID	Non-Steroidal anti-inflammatory Drug
PAS	Periodic Acid-Schiff
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PDR	Pleiotropic Drug Resistance
P-gp	Permeability Glycoprotein
Phe	Phenylalanine
PKC	Protein Kinase C
Pro	Proline
PSC	Posaconazole
R	Resistant
RAPD	Randomly Amplified Polymorphic Deoxyribonucleic acid
REA	Restriction Endonuclease analysis
Rh-6G	Rhodamine 6G
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
S	Susceptible
S-DD	Susceptible Dose Dependent
Ser	Serine
SHAM	Salicylhydroxamic Acid
SI	Staining Index
VRC	Voriconazole
YBC	Yeast Biochemical Card
YPD	Yeast Peptone Dextrose

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CHAPTER I

Introduction

Antifungal Drug resistance and tolerance in Candidaemia patients: from bed side to bench

Introduction

Candida organisms coexists in humans as commensals without damage to the host, colonizing several body locations like the skin, genital tract and gastro-intestinal tract [1] However, as an opportunistic pathogen, whenever the immune status of the host or its microbiota becomes disturbed, it can cause extensive mucosal colonization and disease [2, 3]. *Candida* infections represent an increasing challenge for clinicians. It may range cutaneous or mucocutaneous infections to severe systemic infections. Formerly described in HIV and immunocompromised neoplasic patients, it is now clear that *Candida* may cause serious infections in non-immunocompromised, critically ill and surgical patients [4]. Along the years, in parallel, the advance of medical procedures, the incidence of bloodstream *Candida* infections increased as well as the associated mortality rate [5-7].

One of the main factors that contribute to the high mortality rate associated with *Candida* bloodstream infections is the difficulty in diagnosis, due to the nonspecific clinical symptoms of systemic fungal infection and the delayed laboratorial detection methods, as well as the delay in initiation adequate antifungal therapy [8, 9]. Unlike antibacterial drugs, the array of available antifungals is somewhat scarce. Azoles, polyenes and echinocandins are the main antifungal classes, being the last nowadays considered first-line therapy in many hospitals for the treatment of invasive candidiasis [10-12].

Antifungal prophylaxis with fluconazole seems to have favoured the increase of resistance in yeasts. Despite its recent start of use, increasing reports describe the emergence of echinocandin resistance during treatment, a fact that raises concerns about echinocandin-resistant *Candida* spp [13-19]. The major mechanisms responsible for azole resistance are the upregulation of multidrug efflux transporters which include the ATP-binding cassette (ABC) transporter and the Major facilitators (MF), the alteration or overexpression of the

azole binding site, as well as mutations in the ergosterol pathway [20-29]. Regarding echinocandins, the major mechanisms of resistance are specific mutations in *FKS*1 and *FKS*2 genes that encode essential components of the glucan synthesis enzyme complex [13, 15-17, 25, 30, 31].

With the increase of clinical and/or microbiological resistance, antifungal susceptibility tests play an ever-increasing role in the selection of antifungal drugs [32]. The approved Clinical and Laboratory Standards Institute (CLSI) document M27-A2 2002 (formerly National Clinical Collaborative Laboratory Standards - NCCLS) provides support for the standardization of testing, but still has considerable limitations [33]. It does not provide interpretative breakpoints for all antifungals; it raises problems of trailing endpoints, it is very labour intensive and gives late results. For these reasons most of the clinical laboratories do not follow such a procedure. Particularly in life-threatening situations like fungal sepsis, in patients administered antifungal prophylaxis and in strains isolated from patients who do not respond to treatment, antifungal susceptibility testing is of crucial relevance and should be mandatory [34]. The *in vivo* conditions are significantly different of *in vitro*, in particular, the microorganisms are often under the effect of both antifungal and non antifungal drugs, as is the typical case of critical care patients. The role of concomitant therapies in the promotion of antifungal resistance still remains unveiled.

Throughout this chapter, the main aspects of *Candida* infections such as epidemiology, risk factors, pathogenesis and virulence attributes, antifungal mechanisms of action and resistance will be addressed.

Epidemiology

Candida are opportunistic fungi, nowadays often associated with fatal invasive infections. During the last decade, *Candida* spp infections increased markedly [35-37]. Factors contributing to this trend include a growing population of immunocompromised patients with AIDS, but also of non-immunocompromised critically ill patients submitted to aggressive and invasive therapy [4, 6, 7, 38].

Candida represents the third and the sixth cause of nosocomial bloodstream infections in North America and European ICUs, respectively [39]. In this settings the most common types of Candida infections are bloodstream infections, indwelling catheter-related infections, intra-abdominal infections and urinary tract infections; they are associated with a considerable increase in hospital costs and length of stay [6, 40-43].

The yeast most frequently isolated from ICU patients is *C. albicans* [4, 7, 41, 42, 44-46]. During the 90s novel developments in antifungal prophylaxis strategies in patients at risk, particularly in transplant, hematologic and critically ill patients were made. This contributed to a shift towards a greater involvement of non-*Candida albicans Candida* strains as a cause of candidaemia [6, 7, 46-50]. The drug most frequently used for prophylaxis was fluconazole, which favored the emergence of more resistant species like *C. glabrata*, *C. parapsilosis* and *C. krusei* [39, 46, 48, 51, 52], as well as the emergence of new species including *Saccharomyces cerevisiae* and *Rhodotorula* spp [53-56].

The isolated *Candida* species varies accordingly the clinical settings and the patient population [6]. *C. glabrata* ranks as the second most frequent isolated species from cases of candidemia in the United States [39, 44, 46, 48, 52, 57]. Bloodstream infections by *C. glabrata* occur predominantly in patients with neoplasic disease and associate to high mortality rates [6, 7 ,52]. *C. parapsilosis* stands out as the second most common species isolated from blood in Latin American countries, Asia and Europe and occurs particularly in neoplasic and neonates [6, 7, 41, 58]. Although less prevalent than the other *Candida* spp., *C. krusei* infections raise interest due to its intrinsic resistance to fluconazole and to the fact

that they are more prevalent in elderly patients and with hematologic malignancies [52, 59-61].

A prospective, observational study was conducted at a large Portuguese University hospital, aiming to evaluate the epidemiology of bloodstream fungal infection [7]. The incidence of fungaemia and nosocomial fungaemia during the year of 2004 were 2.7 and 2 per 1000 hospital admissions, respectively [7]. Thirty-five percent of yeast isolates were *C. albicans* followed by *C. parapsilosis* (25.6%). Mortality rate associated with fungemia was 39.3%; the highest values were found in patients yielding *C. glabrata* (78%), *C. tropicalis* (53%) and *C. albicans* (46%) infection [7]. Seventy-five per cent of the fungaemia episodes were nosocomial, with 48% mortality [7]. The main risk factors for an unfavourable fungaemia related outcome included concomitant therapy, the nosocomial origin of the infection and ICU stay [7]. In this study a high percentage (15%) of antifungal resistance was observed; 81% of fungaemia episodes due to resistant strains had been submitted to antifungal treatment (mostly with fluconazole) within the first episode of fungaemia (p=0.017) [7].

Attending to this picture, it was imperative to study the influence of the risk factors involved in patient clinical resistance (unfavourable outcome) in order to manage the high resistance found among us. This was the starting point of this thesis.

Risk factors for candidaemia

Colonization of the skin and mucous membranes and the alteration or disruption of natural host barriers, like wounds, surgery and the insertion of indwelling intravascular catheters are the main predisposing factors for *Candida* infections. Factors like broad-spectrum antibiotherapy, abdominal surgery, presence of central venous catheter, administration of parenteral nutrition and immunosuppressive therapy are the most important risk factors for candidaemia especially in ICU [7, 42, 45]. Among all admissions to the hospital, patients with underlying diseases such as hematologic malignancies or neutropenia, AIDS, extreme ages and those submitted to gastrointestinal surgery, are under an increased risk of

candidaemia [4, 7, 45, 47, 48]. In recent years a trend of increased candidaemia episodes in non-immunosuppressed patients admitted at ICU was registered [45, 62-64]. Among such patients, an important risk factor for the development of *Candida* bloodstream infection is the prolonged stay in the ICU; risk exponentially increases after a length of stay for 7 to 10 days [42, 63, 65].

The ICU setting provides *Candida* the idyllic opportunity for development of infection and subsequent transmission, attending to the fact that most patients are submitted to mechanical ventilation or placed central venous catheters and surgical drainage devices. Fungaemia by *Candida* spp in critical care patients is considered to have in most case an endogenous origin from the gastrointestinal tract [66].

Surveillance strategies have been implemented in order to identify ICU patients at high risk for candidaemia, who may benefit from antifungal prophylaxis or early empiric therapy [67-69]. Ostrosky-Zeichner and co-workers have enrolled 2,890 patients who stayed for more than 4 days in the ICU in order to create a rule that identifies patients at high risk for invasive candidosis [69]. The clinical prediction rule for the early diagnosis of candidaemia in ICU patients used the combination of the following risk factors: any systemic antibiotic or presence of a central venous catheter and at least two of the following, total parenteral nutrition, any dialysis, any major surgery, pancreatitis, any use of steroids, or of other immunosuppressive agents [53]. In 2011 the prediction rule was improved and mechanical ventilation was considered important: mechanical ventilation and central venous catheter and broad spectrum antibiotics and one additional risk factor [70]. Hermsen *et al* recently applied this prediction rule to 352 patients and concluded that it is most useful for identifying patients who are not likely to develop invasive candidosis, potentially preventing unnecessary antifungal use, thus optimizing patient ICU care and facilitating the design of forthcoming antifungal clinical trials [71].

Understanding pathogen distribution and relatedness is essential for determining the epidemiology of nosocomial infections. Establishing clonality of pathogens can aid in the identification of the source (environmental or endogenous) of organisms and distinguish

relapse from reinfection. Many of the species that are hospital-acquired are also common endogenous commensal organisms, and therefore it is important to be able to determine whether the isolate recovered from a patient sample is a pathogenic strain a commensal or a contaminant strain unlikely to be the source of the infection. Molecular typing is a powerful tool in the armamentarium for combating the spread of infection in the hospital environment and to discover the routes of microbial transmission.

Presently, Multilocus sequence typing (MLST) and microsatellite length polymorphism (MLP) are considered the most discriminatory typing methods for *C. albicans*. MLST typing is based on sequence analysis of DNA fragments from six housekeeping genes, *ACC1*, *ADP1*, *GLN4*, *RPN2*, *SYA1*, and *VPS13* [72, 73]. MLST is the typing method more frequently used, mainly because it has a very high discriminatory ability, it has been optimized with a consensus scheme, and is the only typing method that has a public database (http://calbicans.mlst.- net/) where each diploid sequence type (DST) obtained can be deposited and compared with others already available in the database [74]. MLP typing is based on the PCR amplification of microsatellite sequences, defined as tandem repetitive stretches of two to six nucleotides. The PCR fragments obtained after amplification with primers flanking the microsatellite region differ in size according to the number of repetitions of the microsatellite stretch. This technique has been used in several studies addressing *C. albicans* genotyping [75-77]. Recently, the comparison between the ability of MLP and MLST in *C. albicans* typing and grouping indicated that the two methods show similar discriminatory abilities and a high correlation in the clustering of isolates [78]. Randomly amplified polymorphic DNA (RAPD) analysis is another robust typing tool, showing a high degree of discrimination in studies involving nosocomial transmission and microevolution, especially in *C. glabrata* infections [79]. Restriction endonuclease analysis (REA) of the mitochondrial DNA has been described as a valuable tool for *Candida spp*. characterization and has been recently used in order to discriminate between Candida clinical isolates [61, 80, 81].

Antifungal agents: mechanisms of action

The battery of clinical antifungal agents available is limited, in contrast to antibacterial drugs. Limits arise from the number of drug targets in fungi, which are heavily focused in the cell wall and plasma membrane. Nevertheless, pursuit for new cell targets, within the genomic era, has increased exponentially. Throw this section the main antifungal agents used for the treatment of candidaemia will be addressed.

Polyenes

The polyenes belong to a class of natural compounds with a heterocyclic amphipathic molecule (one hydrophilic charged side of the molecule and one hydrophobic, uncharged side). They target ergosterol in the fungal membrane by inserting into the lipid bilayers and creating pores that disrupt plasma membrane integrity, allowing small molecules to diffuse across the membrane resulting in cell death [82]. There are two main polyenes: amphotericin B and nystatin. Amphotericin B is still considered the gold standard in the treatment of most fungal infections, especially in severe invasive infections. However, amphotericin is toxic to mammalian cells, particularly causing nephrotoxicity. To overcome its toxicity a variety of reformulated versions have been introduced. Lipid formulations of amphotericin B are better tolerated than amphotericin B deoxycolate [83]. Although having a broad spectrum activity against most fungi, lipid formulations are very expensive, limiting the use to second-line or salvage therapy.

Pyrimidine analogues

5-Fluorocytosine is the only representative of this class of antifungals. It acts through conversion to 5-fluorouracil by a cytosine deaminase, which is the incorporated into DNA and RNA, inhibiting cellular function and division [82]. Since most filamentous fungi lack cytosine deaminase, the spectrum of flucytosine is restricted to pathogenic yeasts. 5-fluorocytosine is used in combination with other antifungal agents namely amphotericin B, rather than in monotherapy, because resistance develops at high frequency [82].

Triazoles

The triazoles are the largest class of antifungal drugs in clinical use and have been deployed for approximately two decades. They are heterocyclic synthetic compounds that inhibit the fungal cytochrome P450 14α-lanosterol demethylase, encoded by the *ERG11* gene (also known as *CYP51*) which catalyzes the late step of ergosterol biosynthesis. The drugs binds through a nitrogen group in their five-membered azole ring to the heme group in the target protein and block demethylation of the C-14 of lanosterol, leading to the substitution of methylated sterols in the membrane. Inhibition of this enzyme results in decreased membrane ergosterol content and accumulation of toxic methylated intermediates, with resultant disruption of fungal cell membrane function, growth inhibition, and, in some cases, cell death [20, 84, 85]. Triazole antifungal activity is generally fungistatic against *Candida* spp., but fungicidal against *Aspergillus*.

The triazoles include fluconazole, itraconazole, voriconazole and posaconazole. Given its excellent safety and low cost profile and the proven efficacy for the treatment of invasive candidosis, fluconazole remains one of the most commonly used antifungal agents [86]. Voriconazole is a second generation triazole that is active against all *Candida* species and has a broad spectrum of activity and, like itraconazole, is fungicidal against some isolates of filamentous species [87]. Posaconazole differs in structure from the compact triazoles (fluconazole and voriconazole) in part by its extended side chain (a feature held in common with itraconazole); however it displays a dioxolane ring altered to a tetrahydrofluran [84, 88]. The structural differences between the azoles might seem small, but they dictate its antifungal potency and spectrum, bioavailability, drug interaction and toxic potential. Posaconazole is currently only available as oral suspension, and it must be taken with food or a nutritional supplement, somewhat limiting its usefulness. The drug is well tolerated, with an overall safety profile comparable to that of fluconazole [88].

Echinocandins

These compounds are fungicidal *in vitro* against yeasts. However they are not active against *Cryptococcus* spp. Three agents are presently available for clinical use: caspofungin,

micafungin and anidulafungin. They inhibit β-1, 3 glucan synthase, an enzyme complex that is located in the plasma membrane of fungal cells [25, 31, 82, 89]. This enzyme has a minimum of two subunits, Fks1, the catalytic subunit, and Rho, a GTP-binding protein that regulate the activity of the glucan synthase [31]. They are responsible for the production of β-1, 3 glucan which is essential for fungi as they represent one of the major components of the fungal cell wall [31]. The safety profile of echinocandins is excellent, with few reported adverse events and drug interactions. Despite considerably greater cost, echinocandins are replacing fluconazole as the antifungal of choice in ICU setting [86].

Recent studies have shown that echinocandins are efficacious and safe, explaining why these compounds are recommended as the first-line therapy for the treatment of candidemia [90].

Antifungal resistance mechanisms

Patients under long term antifungal prophylaxis or antifungal treatment display favorable conditions for the emergence of antifungal resistance [91].

Three types of antifungal resistance have been described: primary or intrinsic, previous to antifungal exposure, secondary or acquired, and clinical resistance. Secondary or acquired resistance develops following exposure to an antifungal agent and can be either reversible, due to transient adaptation, or persistent as a result of one or several genetic alterations. Clinical resistance relates to patient unfavorable outcome despite antifungal therapy and it is most often to be due to primary or secondary yeast antifungal resistance mechanisms. Factors related to clinical resistance will be focused latter.

Polyenes

Resistance to amphotericin B is quite rare and most often results from mutations in the *ERG3* gene (which encodes a C-5 sterol desaturase, an enzyme involved in ergosterol biosynthesis) and lower the concentration of ergosterol in the fungal membrane [92]. Consequently the accumulation of an alternate sterol in the membrane occurs [92].

Resistance to amphotericin B may also be mediated by increased catalase activity, with decreasing susceptibility to oxidative damage [93]. *C. krusei*, *C. glabrata* and *C. lusitaniae* are less susceptible to amphotericin B [90].

Pyrimidine analogues

The use of flucytosine is nowadays very restricted due to the high prevalence of resistance among clinical isolates and by the speed at which yeast isolates develop resistance under treatment. Resistance of *Candida* clinical isolates correlates with mutations in the enzyme uracil phosphoribosyltransferase (Fur1p) that turns unable the conversion of 5-fluorouracil to 5-fluorouridine monophosphate [20]. The high incidence of 5-fluocytosine resistance recommends its use only in combination with other antifungal drugs like amphotericin B, especially in cryptococcosis [94].

Triazoles

The major mechanism responsible for high level of azole resistance is the overexpression of cell membrane efflux pumps [95, 96]. Two classes of pumps are responsible for lowering the accumulation of azoles inside the yeast cell by actively translocating compounds across cell membrane: ABC pumps and the major facilitator (MF) transporters (figure 1) [1, 9, 21, 24, 27, 34, 45, 46, 61, 66, 97, 98].

The ABC pumps, also called ATP-binding cassette, use the hydrolysis of ATP as energy source. They have low specificity since they accept as substrates azoles but also a wide range of compounds [22]. The most frequently encountered triazole resistance mechanism among clinical isolates is the upregulation or overexpression of mainly *CDR*1 and *CDR*2 genes [99-101]. Their expression is regulated by the zinc finger transcription factor Tac1, which binds to the drug response element (DRE) found in their promoter [102]. CDR expression is increased by gain-of-function mutations in Tac1p, with high level of fluconazole resistance occurring when this mutation is coupled with loss of heterozygosity [103]. Interestingly, *TAC1* is located in the left arm of chromosome 5 (Chr5), the same chromosome where mating-type-locus (*MTL*) is located [104]. *C. albicans* exhibits two MTL alleles, *MTLa* and *MTLα*, and the loss of heterozigoty at MTL locus is frequently associated

with homozygosity at the *TAC1* and *ERG11* loci. This homozygosity is described by some authors to be related to antifungal resistance [23, 103, 105, 106]. However, others showed that homozygosity at MTL is infrequent among clinical isolates and it does not influence directly antifungal resistance [107-109]. Our findings suggest that homozygosity at MTL locus is not frequent among clinical isolates despite the azole resistance pattern, the site of infection or previous *in vivo* antifungal drug exposure (author unpublished data).

As with *C. albicans*, azole resistance in *C. glabrata* clinical isolates is associated with increased expression of PDR ABC drug efflux pumps such as CgCdr1p and Cg Pdh1p, also called CgCdr2p [97, 110]. Contrary to *C. albicans*, *C. glabrata* usually shows high MIC values to azoles, especially fluconazole [111] and prophylaxis with azoles is the main factor responsible for such fact [110]. *C. krusei* shows intrinsic fluconazole resistance, however is susceptible to voriconazole and posaconazole [112, 113]. This innate resistance is due to reduced susceptibility of the drug target Erg11p to azole antifungals [114, 115], however *C. krusei* also possesses efflux pumps namely *ABC1* and *ABC2* [60, 116]. Voriconazole binds more effectively to the cytochrome P450 isoenzyme in *C. krusei* than fluconazole, thus resulting in higher rates of susceptibility [112, 113].

The second main class of multidrug transporters also involved in azole resistance is MF class. *MDR1* gene is involved specifically in resistance to fluconazole rather than other azoles and uses the proton motive force of the membrane as an energy source [117, 118]. The multidrug resistant regulator, Mrr1, is the transcription factor that controls the expression and is upregulated with *MDR1* in drug resistant clinical isolates [27, 118]. The gain-of-function in the transcription factor Mrr1p, followed by loss of heterozygosity, represents the main cause of *MDR1* overexpression in fluconazole resistant *C. albicans* strains [26].

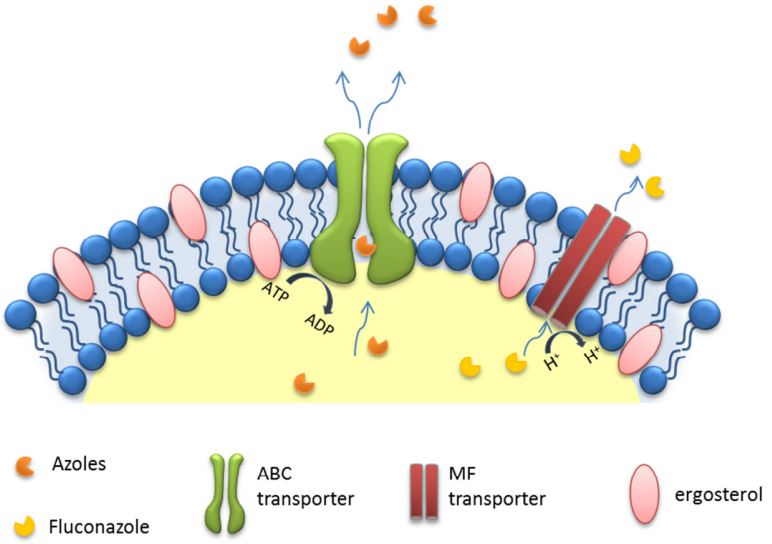


Figure 1. Principal mechanisms of azole resistance by minimizing the impact of the drug in the cell. Upregulation of ABC transporter efflux pumps (ATP dependent) confers resistance to azoles while a major facilitator (MF) transporter (Proton motive force dependent) confers resistance only to fluconazole.

Another mechanism that operates in order to overcome the effect of the drug in the yeast cell is the alteration of the target enzyme Erg11, where at least 12 mutations have been associated with azole resistance, avoiding the binding of the drug to the target [119, 120]. Reduced affinity of Erg11p to azoles seems to be responsible for the intrinsic resistance to fluconazole in *C. krusei* [114, 115]. Upregulation of *ERG11* due to the amplification of the copy number of the gene is another way used by the cell in order to overcome antifungal action [121]. ERG11 overexpression can be achieved through mutations in the transcription factor Upc2 [122]. This transcription factor binds to the azole-responsive enhancer element (ARE) in the ERG11 promoter [123]. Upc2 also binds to two distinct regions on its own promoter to autoregulate expression during azole exposure [28].

Echinocandins

Echinocandin resistance in *Candida* spp. has been attributed to mutations in the *FKS1* gene, the catalytic subunit of β-(1, 3)-glucan synthase, and in a lesser extent in *FKS2*, resulting in amino acid substitutions in conserved regions hot spot 1 (HS1) and hot spot 2 (HS2) (figure 2b) [11]. This mutations turn the mutant enzyme approximately 1,000-fold less sensitive to the drug [31] (figure 2). Acquired mutations in *FKS1* and *FKS2* genes have been predominantly found at position 645 (Serine), S645F (serine to phenylalanine), S645P (serine to proline) and S645Y (serine to tyrosine), and have now been identified in a wide range of *Candida* clinical isolates [31, 124]. Nevertheless the prevalence of Fks mutations in geographically different clinical isolates remains low [18]. Hot spot mutations are more likely to confer resistance to caspofungin than to anidulafungin or micafungin. Such fact suggests that caspofungin could be less potent than the other two drugs [18, 125]. However, these differences in echinocandin potency are abolished in the presence of human serum and therefore cross-resistance is likely to occur *in vivo* [14, 126]. Among *Candida*, MIC values are higher for *C. parapsilosis* and *C. guilliermondii* than for *C. albicans* isolates, although recent reports showed that MIC values are also higher for *C. glabrata*, *C. krusei* and *C. tropicalis* [17, 19, 127]. *C. parapsilosis* exhibits a point mutation at amino acid position 660 resulting in a proline to alanine substitution, which is thought to be responsible for the intrinsically less susceptible profile to caspofungin [30]. *C. guilliermondii* displays three amino acid polymorphisms in the first hot spot region in Fks1 and Fks2 [31].

Echinocandin treatment may trigger cell wall salvage mechanisms producing physiological alterations that decrease the susceptibility to these antifungal agents [128]. The inhibition of the β-(1, 3)-glucan synthesis leads to a compensatory increase in chitin synthesis (figure 2c) mediated by the PKC cell wall integrity MAP kinase, Ca^{2+} - calcineurin and High Osmolarity Glycerol Response (HOG) signaling pathways [129]. This increase in chitin content is responsible for the paradoxical growth or “eagle effect” and occurs most frequently with caspofungin than with anidulafungin and micafungin [130-132]. There is now evidence that the compensatory elevated chitin content is likely to occur also *in vivo* [133].

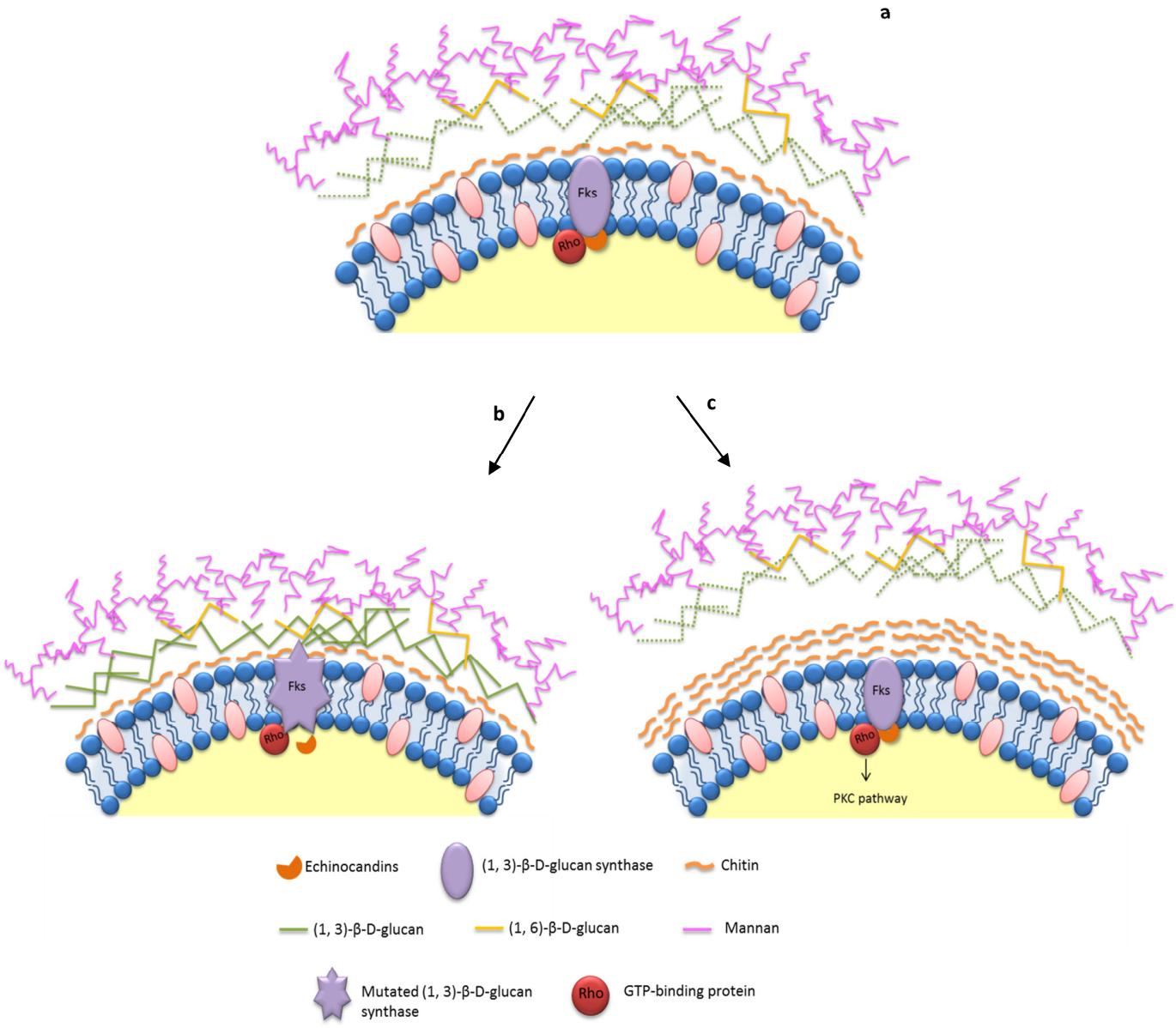


Figure 2. Mechanisms of echinocandin resistance and tolerance. a) The disruption of (1,3)-β-D-glucan by echinocandins causes a loss of cell wall integrity and severe stress to the fungal cell. b) Mutations of (1,3)-β-D-glucan synthase confers resistance to echinocandins by minimizing the impact of the drug in the cell. c) Another way to overcome echinocandin action is by triggering a stress response through Rho1, a positive regulator of glucan synthase, which activates PKC, calcineurin and HOG pathways, producing an upregulation of chitin synthesis.

Methods for assessing antifungal drug resistance

The emergence of resistant strains to antifungals renders imperative the routine evaluation of the susceptibility pattern to antifungal agents, a test that is not routinely performed in most laboratories.

The ideal method for susceptibility testing should include the following requisites: easy to perform, fast, reproducible, cost effective and ability to detect fungal isolates that exhibit decreased susceptibility profile or resistance to antifungals. Early detection of fungi in blood or other specimens with a rapid assessment of drug susceptibility could improve the survival of patients with invasive disease by accelerating the initiation of appropriate antifungal treatment.

Laboratory assessment of antimicrobial susceptibility is often regarded as a prerequisite for correct therapeutic management of infectious diseases. However antifungal susceptibility tests are not always accurate predictors, since they not take into account the dynamic and complex biology of fungi exposed to an antifungal *in vivo*. They are based on the measurement of the reduction of microbial growth resulting from the exposure to an inhibitor. Various testing procedures include broth microdilution, agar and disk diffusion and Etest®. These techniques vary in cost, accordance between methods, reproducibility and interpretation [134, 135].

Standardization of susceptibility testing methodology between laboratories is a basic requirement to ensure compatibility of susceptibility data. The US National Committee for Clinical and Laboratory Standards (NCCLS, now the CLSI – Clinical Laboratory Standard Institute) [33] has developed standardized methods for susceptibility testing. The most widely used quantitative susceptibility tests estimate the minimal inhibitory concentration (MIC) of an antimicrobial. The ability to generate a MIC is of little value without the corresponding ability to interpret its clinical meaning. Like in the case of antibacterial agents, breakpoints can be established for antifungal agents based on a number of factors including distribution curves of MIC values for wild-type populations of particular organisms, as well as their pharmacokinetic and pharmacodynamics properties [136]. CLSI resistance breakpoints are based on data relating treatment outcome to antifungal MIC

values, and indicate the MIC at which clinical responses showed a marked fall-off [137]. They do not serve as absolute predictors of clinical failure or success, since *in vitro* resistance does not always result in clinical failure or an *in vitro* susceptibility profile not always correlates to a favorable clinical outcome [34]. Furthermore MICs do not distinguish cidal from static drug activity.

CLSI proposes a reference protocol as a basis for standardized antifungal susceptibility testing regarding yeasts, the M27 A3 protocol. However this methodology still has considerable limitations. Although M27 A3 protocol has now defined breakpoints for most antifungals, including echinocandins, they lack information regarding posaconazole and amphotericin B [33]. Also, when using azole susceptibility tests, the trailing growth represents an additional problem for interpretation of susceptibility endpoints [134, 138]. Besides, this protocol is just based on growth assays, giving no information about the possible mechanisms of resistance involved. Pfaller *et al* recently defined the "epidemiological cutoff values" (ECVs) for the interpretations of *in vitro* susceptibility testing results. The future application of ECVs will be important for the detection of emergence of resistance to azoles and echinocandins and will also represent an important step forward the development of improved species-specific clinical breakpoints [139-141].

Over the last years microbiology laboratories witnessed considerable changes, with the development of more accurate techniques, not only in clinical routine but also in research. Flow cytometry (FC) represents an efficient and fast approach for the analysis of cell architecture and functional phenotypes, with considerable advantages over conventional methods. FC has been recognized as a possible tool for antifungal susceptibility testing, principally of *Candida* spp. [142-144]. By using suitable dyes it is possible to perform a rapid detection of damaged fungi and to examine the nature of drug-induced damage to yeasts [142-146]. This methodology allows the timely determination of susceptibility patterns with excellent correlation with the CLSI reference susceptibility testing method [142-144]. Besides the antifungal susceptibility profile, flow cytometry can predict the mechanism of resistance involved. Pina-Vaz et al. showed that flow cytometry analysis using FUN-1

staining provides not only information regarding *Candida* metabolic activity but also additional information about the mechanisms of azole resistance, being a good marker of efflux activity [145, 147]. The measurement of intracellular accumulation of Rh-6G is another helpful method to identify azole resistance due to efflux pumps using flow cytometry [103, 148].

Recent years have witnessed the growth of molecular biology technology, ideally suited for fungal identification and assessment of drug resistance mechanisms. Real-time PCR and sequentiation techniques have been widely used for the quantification of gene expression and search for transcriptional regulator mutations involved in the evolution of antifungal drug resistance [13, 97, 101-103, 149, 150].

Within the microarray area, the transcriptome of the complex yeast network unveiled the genetic mechanisms responsible for triazole and echinocandin resistance in *Candida* spp [23, 60, 149, 151-153]. Besides transcriptome, protein microarray technology, allows the assessment of protein-protein, protein-DNA, protein-small molecule interaction networks as well as post-translational modification networks in a large-scale [154, 155]. Proteomics analysis has also been used to study the adaptive response of *C. albicans* to azole, polyene and echinocandin and to identify changes in protein abundance in matched sets of azole susceptible and resistant clinical isolates of *C. albicans* [156-159].
All these methodologies provide an opportunity to develop molecular diagnostic platforms suitable for rapid detection of primary and secondary antifungal drug resistance, as well as novel tools for the discovery of new targets and therefore the design of new therapeutic protocols.

Risk factors contributing to clinical resistance: patient *versus* yeast *versus* drugs

For the clinicians, the three main issues of concern about antifungal resistance are: how commonly does it occurs, how easy it is to induce through inappropriate usage of antifungal agents, and how often does it result in failure of treatment. Clinical resistance may be defined as the persistence or progression of an infection despite appropriate antifungal

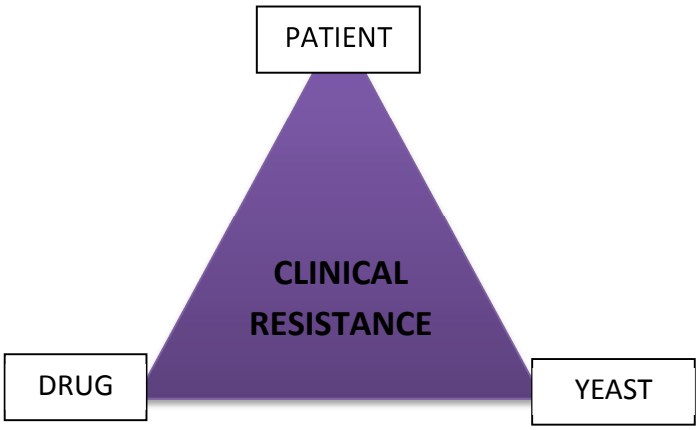
therapy and of an *in vitro* antifungal susceptibility profile. Many factors may contribute to clinical resistance and to the discrepancy between the laboratory susceptibility pattern and the clinical outcome (figure 3). The antifungal efficacy is soaked into a Bermuda triangle that goes through patient, drug and yeast factors that ultimately are responsible for the clinical outcome (figure 3).

Under stressing conditions like antifungal exposure, *Candida* cells may exploit several cellular responses, such as development of mutations, overexpression of multidrug efflux pumps, modulation of Ca^{2+}- calmodulin-calcineurin or the cAMP protein kinase A pathways [160]. Given its clinical relevance, *C. albicans* has been the subject of extensive research in order to unveil the mechanisms governing fungal virulence and drug resistance. The success of this yeast as a pathogen depends largely on its ability to generate diversity not only at the genetic level but also at the morphological and physiological level.

The mitochondrial respiratory pathway can regulate the metabolic behavior contributing to fitness and flexibility of *Candida* strains in response to external challenges [161, 162]. The existence of an alternative respiratory pathway is present in some Candida species, especially *C. albicans* and is implicated in reduced susceptibility to azoles [161-163].

C. albicans is considered to be pleomorphic due to its ability to switch from yeast to hyphal or pseudohyphal form [164]. Morphogenesis changes are coupled to biofilm formation, which plays an important role in virulence. *C. albicans* can produce biofilms on medical implants, like indwelling vascular catheters, and its formation acts as a physical barrier, protecting the underlying cells of being exposed to antifungal drugs, hence lowering the available drug concentration [165]. In addition, efflux pumps can be upregulated during biofilm formation which may confer increases in resistance to azoles [166, 167].

- Pharmacogenomic
- Immunity
- Age
- Gender
- Physiological factors (body size, gastrointestinal physiology, etc…)
- Pathological conditions
- Environmental status (diet or pollutants)



- Pharmacokinetics (gastrointestinal pH, efflux pumps, plasma proteins, lipoproteins)
- Concomitant therapy (fluoroquinolones, rifampicin)
- Fluconazole prophylaxis
- Chemical properties

- Phenotypic switching
- Morphogenesis
- Biofilms
- Calcineurin pathway
- HSP90
- Resistance mechanisms
- MAPK (Mitogen-activated protein kinase): HOG and PK pathways
- Genome plasticity (aneuploidy)
- Site of infection

Figure 3. Risk factors that contribute to clinical resistance. Information was collected from the following references: [6, 9, 21, 34, 41, 52, 54, 121, 128, 160, 164-166, 168-170].

Concomitant medications administered to patients, such as antibiotics, can influence the pharmacodynamics of the antifungals. Fluoroquinolones antagonize fluconazole activity against *C. albicans* strains [170], whether rifampicin can induce the expression of MDR1 pumps [168]. Nevertheless, the effect of other medications, some of them life-saving in the case of critical care patients still remains to be elucidated.

The choice of an antifungal agent for the empirical treatment of *Candida* bloodstream infections is a complicated task. Similarly to the findings with the extensive use of antibiotics and the development of multiresistant bacterial pathogens, the selective pressure due to the widespread use of fluconazole in prophylaxis, promoted a shift toward non-*albicans Candida* species, like *C. glabrata* and *C. krusei* [171, 172].

Patient pharmacogenomics, which can influence drug absorption, distribution and metabolism, its immunological status and the underlying disease are additional important factors to be considered when managing individual patients [173].

Strategies to defeat antifungal resistance

The knowledge of the mechanism of antifungal resistance brought by the genomic era supports the development of therapeutic strategies in order to bypass drug resistance. The principal cell mechanism of antifungal resistance is the active transport of drugs out of the cell by efflux pumps [24, 27, 98, 101], expressed not only by yeasts but also by humans cells [21, 173]. The main strategy to reduce efflux impact involves the maintenance of a high antifungal concentration inside the cell, at its site of action. The simplest approach would be the use of antifungals that are not substrate of efflux pumps, like amphotericin B or echinocandins, which given their hydrophobicity and size, do not interact with the efflux pump [25, 174].

The second approach would be the development of inhibitors or chemosensitizers of efflux, affecting the target, the activity, by blocking access to the binding site, or even the efflux pump transcription.

In humans, one of the factors that is responsible for the failure of cancer therapy are ATP-dependent drug efflux pumps, such as P-glycoprotein (P-gp) [175]. P-gp substrates such as FK506 [176] or cyclosporine A (CsA) [177] are immunosuppressors that are able to inhibit efflux. They act similarly in *C. albicans* strains, inhibiting the calcineurin-mediated azole tolerance by binding to small, abundant, conserved binding proteins called immunophilins. CsA binds with cyclophilin A (Cyp1p) and FK506 with FKBP12, to form protein-drug complexes that inhibit calcineurin [24, 169, 178]. By inhibiting calcineurin these compounds act synergistically with azoles [169, 179, 180]. While FK506 and CsA chemosensitize *C. albicans* cells to azoles, rending the azoles fungicidal, they are also immunosuppressive drugs, which make it administration problematic in immunosuppressive candidosis patients. Nevertheless, the inhibition of calcineurin-mediated azole tolerance is still a potential therapeutic approach [181]. Non-immunosuppressive analogs could inhibit fungal calcineurin by exploiting structural differences between the human and the fungal targets [181].

Ibuprofen ([2-(4-isobutylphenyl)-propionic acid has been described to act synergistically with pyrazinamide [182], fluconazole [101, 145, 183] and amphotericin B [184] in fungi. In *C. albicans* expressing *CDR* efflux pumps, the presence of ibuprofen increased azole intracellular accumulation, changing the resistant phenotype to susceptible [101, 145]. This potent anti-inflammatory, non-steroidal drug might play important role in future therapeutic strategies. However, its *in vivo* effect still remains unveiled.

Another helpful strategy would be the design of inhibitors that could act indirectly on efflux, de-energizing the ATP or H^+ dependent transporter, by lowering the cytoplasmic ATP concentration or depleting the electrochemical potential of the plasma membrane, respectively [22, 185]. However, by altering ATP and membrane potential, other cellular metabolic activities could be compromised. Alternatively, the promotion of antifungal uptake could also be a strategy to overcome antifungal resistance due to efflux. Dubikovskaya *et al*. showed that the inclusion of multiple arginine residues (octaarginine

[R8]) in human anticancer drugs enhances the delivery to its intracellular targets [186], an approach that has already been tried in yeasts [185].

The medical complexity of patients taken together with the intricate cellular mechanism involved in drug resistance makes the pursuit of effective solutions mandatory.

CHAPTER II

Aims

Aims of the Study

This study has the following goals:

1. Characterization of yeasts isolates from fungaemia patients in order to assess: the source of infection and modes of transmission; the genetic relatedness and the antifungal susceptibility profile;
2. To evaluate the role of chitin in echinocandin resistance and to develop of a new methodology for evaluating chitin content in the fungal cell wall;
3. To characterize the *in vivo* mechanisms enrolled in the induction of resistance during echinocandin treatment;
4. To evaluate the role upon an alternative respiratory pathway in *C. krusei* as a possible contribution to resistance to cell stresses;
5. To unveil the effect of concomitant therapies used in critical care patients upon antifungal resistance or tolerance, like propofol or vasoactive amines, such as adrenaline and noradrenaline;
6. To assess the *in vivo* reversion of azole resistance by ibuprofen, in an animal model of systemic *Candida* infection.

CHAPTER III

Results

Part I

Genetic relatedness and antifungal susceptibility profile of *Candida albicans* isolates from fungaemia patients

Background

Candida infections have progressively emerged as major health care related invasive fungal infections since the late 80s, mainly arising from an endogenous source, either digestive or mucocutaneous. Commensalism, followed by colonization, usually precedes dissemination, most frequently in patients with transient or permanent immunocompromised status, such as transplant recipients, chemotherapy patients, underweight neonates and human immunodeficiency virus-infected individuals. Significant morbidity and mortality rates have been associated to bloodstream infections due to *Candida* spp [7, 187, 188].

Several polymorphic microsatellite loci have been identified in the genome of *C. albicans* near *EF3*, *CDC3* and *HIS3* [75] or inside the coding regions of *ERK1*, *2NF1*, *CCN2*, *CPH2*, and *EFG1* [189]. However the discriminatory power for each locus is relatively low. In order to more rapidly obtain a higher discrimination, simultaneous amplification of sets of microsatellite markers can be performed. A multiplex system with a high discriminatory power was recently described and found to represent an efficient molecular tool for the swift and accurate differentiation of *C. albicans* [76, 190].

During a twelve month period (2004) a prospective study addressing fungaemia was conducted at Hospital de São João, a large university hospital located in the Northern region of Portugal [7]. The epidemiological data analyzed included the department of admission, underlying diseases and antimicrobial therapy, among others. Several yeast isolates from blood cultures were collected and analyzed as well as fungal strains isolated from surveillance cultures or from medical indwelling devices. These included isolates from distinct biological sources for the same patient, such as urine and lower respiratory

secretions, and from central venous catheters. All episodes of recurrence were investigated [7].

Our main purpose was to genotype all *C. albicans* isolates by using a multiplex PCR system with four microsatellite loci. Ultimately, we aimed to determine the genetic relatedness between simultaneous and/or recurrent isolates from the same patient, the source of infection, the route of transmission, as well as the possibility of transmission among distinct patients. Additionally, the susceptibility profile of all isolates was determined and analysed in regard to antifungal therapy.

Materials and Methods

Patients clinical data

Thirty five *C. albicans* isolates from the blood and other biological sources were collected from twelve patients with fungaemia. The data documented included patient gender and age, department and date of admission, concomitant therapy (i.e. immunossupressors), date and site of fungal isolation, and nosocomial origin of the fungaemia. According to the Centre for Disease Control and Prevention (CDC), nosocomial fungaemia was identified whenever a patient yielded at least one fungal positive blood culture following 48 hours of hospital admission [191]. Antifungal therapy (prophylactic or therapeutic) and clinical outcome (survival/death) were also documented. Fungaemia outcome was evaluated 30 days after the first fungaemia episode. Fungaemia-related death was defined as death occurring within 30 days after the first fungal positive blood culture, with no signs of intracerebral or gastrointestinal bleeding or pulmonary embolism. All *Candida* isolates were frozen at –70ºC in Brain-Heart medium with 5% of glycerol (Difco) and subcultured twice in agar Sabouraud (Difco) prior to experimental procedures.

C. albicans genotyping

Yeast strains were grown overnight in Sabouraud broth at 30ºC. A Zymolyase-based method was used to extract DNA, as previously described [192]. To assess strain relatedness, all isolates were genotyped using a microsatellite multiplex PCR assay with three markers (CAI,

CAIII and CAVI) as described by Sampaio *et al*. [76] and a singleplex amplification reaction assay using the microsatellite marker CEF3, according to procedures described by Bretagne *et al* [75]. Following PCR amplification, a 1 to 2-µl aliquot of each sample was added to 15 µl of formamide containing 0.4 µl of GeneScan^{TM} - 500 TAMRA size standards (Applied Biosystems). Amplicons were denatured at 95°C for 5 min and immediately placed on ice. Denatured samples were resolved by capillary electrophoresis in an ABI Prism 310 genetic analyzer (Applied Biosystems). Determination of allele sizes was automatically performed with GeneScan 3.7 analysis software. The alleles were designated according to the number of repeated units for the CAI ($(CAA)_2CTG(CAA)_n$), CAIII ($(GAA)_n$) and CAVI ($(TAAA)_n$) markers and by the number of nucleotides for the CEF3 ($(TTTC)_n(TTC)_n$) marker.

Antifungal susceptibility profile

Antifungal susceptibility testing of all isolates was performed according to the CLSI M27 A3 protocol in RPMI 1640 (Sigma) [33]. Minimal inhibitory concentration (MIC) for fluconazole, voriconazole, caspofungin and amphotericin B were determined [33]. The yeast strains were classified as susceptible (S), susceptible-dose dependent (S-DD) and resistant (R) to azoles according to the breakpoints defined by CLSI [33]. Although definitive breakpoints have not yet been established for amphotericin B, strains showing MIC ≤1 µg/ml were considered susceptible [193]. For caspofungin, a MIC ≤2 µg/ml corresponded to S and >2 µg/ml to non-susceptible (NS) strains [33]. Strains displaying a MIC variation within one dilution were considered as having a similar susceptibility pattern.

Results

The patients age ranged from 40 to 83 years old and 75% (9/12) were male. Data such as department of admission, date of first isolation and strain origin are presented in table 1 and figure 1. All patients were iatrogenically immunosuppressed, and 75% died. All fungaemia cases corresponded to episodes of nosocomial infections. The microsatellite genotyping analysis is detailed in Table 1.

Amongst the 12 patients two or more isolates were obtained from blood and from

additional body locations. We considered isolates has been highly related when their genotypes differed at a single locus only. In the large majority of patients, blood isolates displayed the same multilocus genotype or a genotype that was highly similar to that of isolates from other body locations. In one single case, patient 10, one isolate from urine exhibited a completely distinct multilocus genotype from the blood isolate, even though they were collected at the same time. Isolate 9.3 from urine displayed a genotype that differed at two loci compared to the isolates obtained from the respiratory tract or blood isolates. This isolate was moderately related to the other isolates from the same patient but was highly related to isolates from patients 6, 7 and 8. In two patients, 11 and 12, minor genetic differences were detected among blood isolates and isolates from other body sources. In these patients, the isolates were highly related, suggestive of microevolution.

Consecutive blood isolates from the same patient exhibited the same microsatellite genotype in 5 out of 6 patients. The exception was for patient 12 where a highly related isolate was observed between two consecutive blood cultures. This was indicative of strain maintenance regardless of antifungal treatment rather than re-infection by a distinct strain (table 1). Isolates from patients 3, 4 and 5 showed identical or highly similar microsatellite profiles (table 1), an interesting fact since these patients had been initially simultaneously admitted in the same intensive care unit (ICU). Isolates from patients 6, 7 and 8 also exhibited identical or highly similar genotypes (table 1) and were initially admitted to the same ICU, although not concurrently. All isolates from patients 1 and 2, whom where in the same ICU in the previous months, displayed the same genotype (table 1). Sharing of the same strain among different patients is suggestive of hospital-acquired exogenous nosocomial infection with hospital endemic strains.

The antifungal therapy timelines are detailed in figure 1. All doses were administered according to the Clinical Practice Guidelines for the management of Candidosis [10]. Four patients (1, 3, 5 and 9) were under antifungal prophylaxis with fluconazole for 2 or 3 weeks prior to the first fungal isolation. Only patient 1, in which fluconazole therapy was replaced by amphotericin B, had a favorable outcome. Two patients (patient 3 and 6), with end-stage

neoplasic diseases, were infected with fluconazole-susceptible strains and died due to fungaemia according to their medical records. In patients 3, 4 and 5 infected by the same strain, MIC increases were observed. Isolates from patients 4 and 5 that were collected several months after isolates from patient 3 were resistant to azoles (Table 1 and Figure 1). Similarly, we observed azole cross-resistance in isolates from patient 8 that were collected several months after isolates from patients 6 and 7. All of these isolates had identical genotypes and probably represented the same strain (Table1 and Figure 1).

All of the isolates analyzed showed low MIC values to amphotericin B and caspofungin (≤1 μg/ml) (Table 1).

Table 1. *Candida albicans* isolates from blood cultures and from other body sites obtained from 12 patients and respective multilocus genotyping results.

Patient	Isolate no.	Source	Isolation date(day/ month)	Microsatellite genotype				MIC mg/ml				
				CAI	CAIII	CAVI	CEF3	FLC	ITC	VRC	AmB	CAS
1	1.1	Blood	21/4	11-11	5-6	7-11	129-143	>64	>8	>8	1	0.5
	1.2	Bronchial washing fluid	22/4	11-11	5-6	7-11	129-143	>64	>8	>8	0.5	1
2	2.1	Lower respiratory secretions	15/4	11-11	5-6	7-11	129-143	>64	4	>8	0.5	0.5
	2.2	Urine	15/4	11-11	5-6	7-11	129-143	>64	4	>8	0.5	0.5
	2.3	Blood	16/4	11-11	5-6	7-11	129-143	>64	4	>8	0.5	0.5
3	3.1	Central venous catheter	02/4	26-28	6-6	7-7	136-145	0.125	0.015	0.015	0.25	1
	3.2	Blood	22/4	26-28	6-6	7-7	136-145	0.125	0.015	0.015	0.25	1
	3.3	Urine	25/4	26-28	6-6	7-7	136-145	0.125	0.015	0.015	0.25	1
	3.4	Blood	30/4	26-28	6-6	7-7	136-145	0.125	0.015	0.015	0.25	1
4	4.1	Blood	30 /8	26-26	6-6	7-7	136-145	32	>8	>8	0.5	0.25
	4.2	Urine	30 /8	26-26	6-6	7-7	136-145	32	>8	>8	0.5	1
	4.3	Blood	08 /9	26-26	6-6	7-7	136-145	32	>8	>8	0.5	1
5	5.1	Blood	20 /9	26-26	6-6	7-7	136-145	>64	>8	>8	1	1
	5.2	Lower respiratory secretions	20 /9	26-26	6-6	7-7	136-145	>64	>8	>8	1	1
6	6.1	Blood	14/4	25-25	6-7	9-14	131-131	0.25	0.015	0.015	0.25	0.25
	6.2	Blood	26/4	25-25	6-7	9-14	131-131	0.25	0.5	0.03	0.5	0.5
7	7.1	Blood	11/7	21-25	6-7	9-14	131-131	0.5	0.03	0.015	0.25	0.25
	7.2	Blood	19/7	21-25	6-7	9-14	131-131	0.5	0.03	0.015	0.25	0.5
8	8.1	Blood	22/10	21-25	6-7	9-14	131-131	>64	>8	>8	0.25	1
	8.2	Pleural fluid	25/10	21-25	6-7	9-14	131-131	>64	>8	>8	0.5	0.5
9	9.1	Blood	26/10	21-26	7-7	9-14	130-131	16	0.06	0.25	0.5	1
	9.2	Blood	05/11	21-26	7-7	9-14	130-131	16	0.06	0.25	0.5	1
	9.3	Urine	08/11	21-25	6-7	9-14	130-131	16	0.06	0.25	0.5	1
	9.4	Lower respiratory secretions	08/11	21-26	7-7	9-14	130-131	16	0.06	0.25	0.5	1
10	10.1	Blood	02/8	11-17	5-5	7-7	126-135	>64	4	>8	0.5	1
	10.2	Urine	02/8	27-46	6-11	20-20	139-144	>64	4	>8	0.5	1
11	11.1	Blood	21/9	29-29	6-6	7-11	129-143	>64	>8	>8	1	1
	11.2	Urine	21/9	28-29	6-6	7-11	129-143	>64	>8	>8	1	1
12	12.1	Catheter	18/5	18-34	6-7	40-40	137-139	>64	2	>8	0.5	1
	12.2	Blood	31/7	18-34	6-7	42-42	137-139	>64	4	>8	0.5	1
	12.3	Lower respiratory secretions	31/7	34-34	6-7	40-40	137-139	>64	0.5	0.03	0.5	0.5
	12.4	Blood	26/8	18-34	6-7	40-40	137-139	>64	>8	>8	0.25	0.25

Strain replacement is highlighted in dark grey
Microevolutionary events are highlighted in light grey

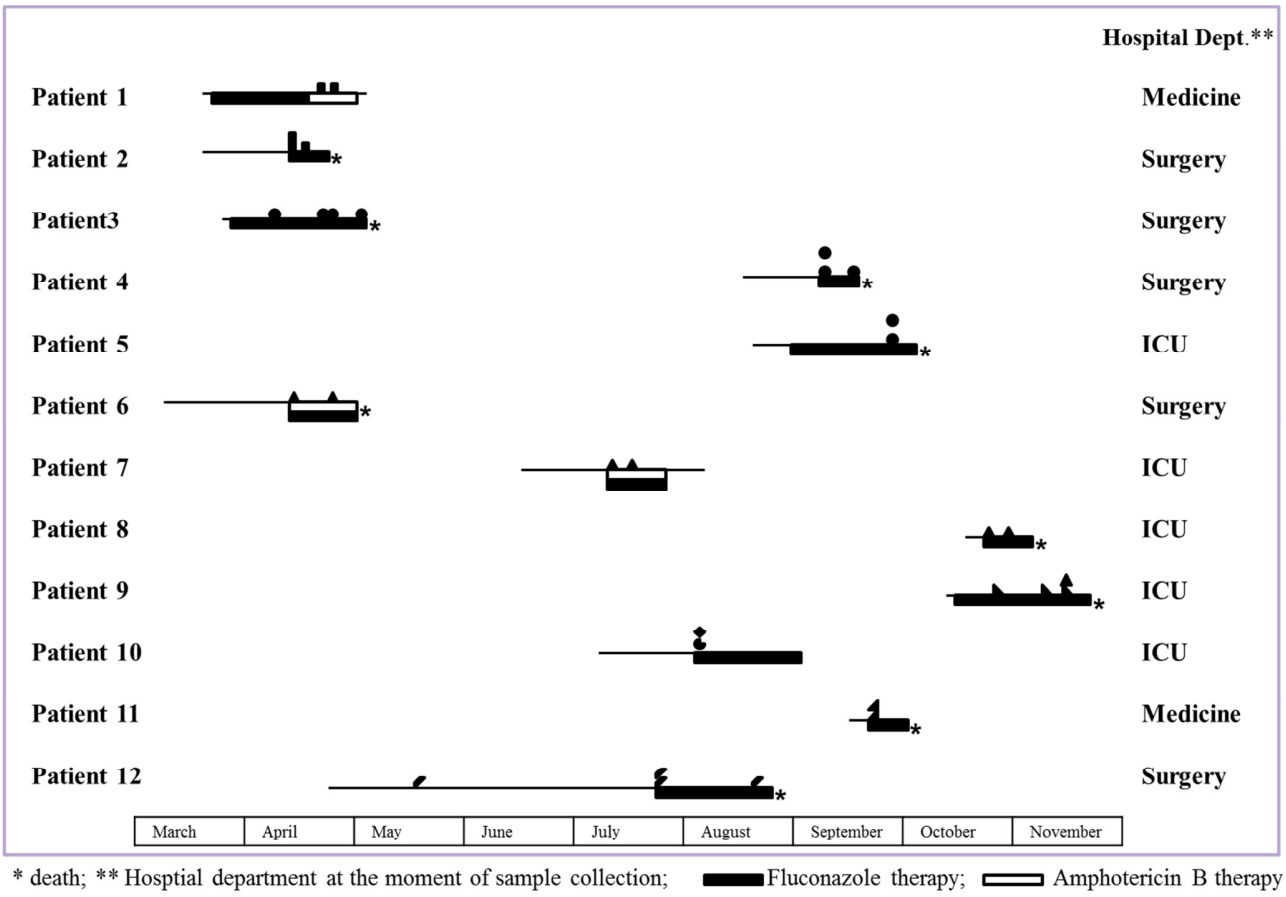


Figure 1. Schematic representation of patients clinical data and genetic relatedness of *C. albicans* strains. The duration of hospitalization (from admission to discharge or death) of each patient is represented by a thick line. Identical symbols represent isolates with the same or highly related genotype.

Discussion

Nosocomial infections represent an important source of morbidity and mortality in hospital settings [194]. The understanding of pathogen distribution and relatedness is critical for both the epidemiological surveillance of health-care related infections and for the conception of rational pathogen control policies [195]. Pathogen typing allows for the determination of genetically and epidemiologically related isolates. The development and implementation of new DNA based technologies and molecular analyses over the last 3 decades have led to considerable advances in microbial typing approaches [77, 196, 197]. The identification of a pathogen origin, endogenous *versus* exogenous, and the characterization of consecutive infections by the same organism as relapse or re-infection are critical [197]. Our group has previously developed a microsatellite multiplex PCR strategy with high discriminatory power for typing *Candida albicans* [76]. This methodology was found to efficiently discriminate *C. albicans* strains, as well as *C. glabrata*, *C. parapsilosis* and *C. tropicalis* [77, 198, 199]. Maintenance and infection by the same strain may indicate that the therapeutic regimen was unsuccessful and that alternative therapies might be required. The results obtained here for successive blood isolates from the same patient suggest the failure of antifungal therapy in these cases. Moreover, fungal strains may invade the host from different body sites, and microevolution, due to strand slippage during DNA replication in the microsatellite region or loss of heterozygosity (LOH), may represent an adaptive fungal response to new environments [76, 196]. We detected microevolution events in 3 patients (9, 11 and 12). *C. albicans* colonization and/or infection of different body sites may represent a predisposing condition or an initial step towards the subsequent fungaemia development [188, 200].

All the fungaemia cases considered herein were of nosocomial origin, according to the accepted definition [191]. Our results clearly demonstrate that isolates displaying the same or highly similar genotypes were obtained from patients who shared the same hospital department of admission. This suggests hospital-acquired infections. These observations indicate that the safety measures between patients most probably failed and those

incidents of cross-infection were likely to have occurred.

In our study, fungaemia relapses were frequent, appeared to be caused by the same strain, and were invariably associated to a poor therapeutic outcome. The extension of cross-resistance to azoles detected among fungaemia patients may challenge the large empiric use of fluconazole and how it can lead to the development of dramatic resistance. We observed induction of azoles resistance in two groups of patients (patients 3, 4 and 5 and patients 6, 7 and 8) and the increase in MIC values, over time, in similar strains from different patients suggests that the strains were endemic to the hospital environment for at least several months. This exemplifies the risk of selecting for strains with increased antifungal resistance in the hospital environment.

The molecular identity of fungal isolates represents a key feature for comprehensible therapeutic strategies in a near future; indeed, analysis of dominant genotypes in different geographical regions, distinct clinical samples and distinct underlying diseases is now a possibility. Molecular approaches are now available, allowing detailed comparisons between *C. albicans* clinical strains and providing a clear definition of their genetic relatedness.

Part II

Determination of chitin content in fungal cell wall: an alternative flow cytometric method

Background

Chitin is a β-1,4-homopolymer of *N*-acetylglucosamine that is synthesized by chitin synthase enzymes [201]. This polysaccharide is present in most fungi and together with β-1,3-glucan, plays a fundamental role in maintaining fungal cell integrity and conferring structural rigidity during growth and morphogenesis [201-203]. Mutations in glucan synthase genes reduce glucan levels in the cell wall while stimulating salvage pathways leading to increased chitin synthesis. This pathway restores the strength of the cell wall matrix and prevents antifungal action [204]. Since chitin is not present in human cells, inhibition of chitin synthesis has been proposed as a potential, selective antifungal target.

The assessment of cell wall chitin content based upon glucosamine release through acid hydrolysis has been used extensively; however this method is very laborious and time consuming [128, 129, 205]. Epifluorescence microscopy has also been widely used to quantify chitin levels in fungi stained by Calcofluor White (CFW), a specific chitin dye [128, 206-210]. However, with this approach, only a limited number of yeast cells can be analyzed and the quantification of the fluorescence emitted cannot be performed accurately [128, 211, 212]. Flow cytometry represents an efficient and fast approach for the analysis of cell architecture and functional phenotypes, with considerable advantages over conventional methods [103, 145, 147, 213].

Here we describe a fast and reliable protocol to measure cell wall chitin content in yeasts cells based upon flow cytometric analysis after CFW staining.

Material and methods

Strains

Twenty two *Candida spp* and 4 *Cryptococcus neoformans* clinical isolates with well characterized susceptibility profiles to caspofungin (antifungal chosen as representative of echinocandin class), were used in this study (detailed in Table 1). SC5314 [214] with wild type chitin levels, *chs3*Δ/*chs3*Δ (Myco 3) [215], *pga62*Δ/Δ [216] and *pga31*Δ/Δ [216] were used as control strains.

Measurement of cell wall chitin content

Wild type and mutant yeast cells were grown in YPD broth medium at 35ºC, 150 rpm, until late logarithmic phase, and used to optimize flow cytometric protocol. A 10^6 yeast cells ml^{-1} suspension in sterilize distilled water was stained with 0 (autofluorescence), 2.5, 6.25, 12.5 and 25 µg CFW ml^{-1} (Fluka, St. Louis, USA), a specific chitin dye (excitation at 365 nm and emission at 430 nm), for 15 minutes at room temperature. In parallel, yeast cells were treated with MIC values of caspofungin during 2 hours, and stained with CFW. The yeast cells were washed twice and blue fluorescence (Pacific blue channel) emitted by 50000 cells was quantified, using a BD FACSCanto™ II (Becton Dickinson, San Jose, CA, USA) flow cytometer. BD FACSCanto™ II system consists of an excitation source with three lasers: blue (488-nm, air-cooled, 20-mW solid state), red (633-nm, 17-mW HeNe), and violet (405-nm, 30-mW solid state). The mean intensity of fluorescence (obtained from three independent experiments) emitted from stained (positive population) and non-stained (autofluorescence or negative population) yeast cells was analyzed and processed with FACSDiva software (version 6.1). In each experiment a staining index (SI) was calculated as follows: (mean intensity of fluorescence of positive population – mean intensity of fluorescence of negative population)/ 2 x standard deviation of the mean intensity of fluorescence of negative population [217]. The chitin content of the 26 clinical isolates was assessed according to the described protocol, after staining with 2.5 µg CFW ml^{-1}; the SI was calculated.

Epifluorescence microscopy

In order to confirm flow cytometry results, epifluorescence microscopy analysis was performed. Yeasts cells were grown and prepared as described for flow cytometric assays and stained with 25 µg CFW ml^{-1} for 15 minutes. Following staining, 30 µl of the cell suspension were placed on a glass slide and overlapped with vectashield fluorescence mounting medium (Vector Laboratories, Peterborough, UK) and observed under an epifluorescence microscope (400X) imager Z Apotome (Zeiss, Barcelona, Spain).

Paradoxical effect of caspofungin

The ability of the clinical isolates to grow in the presence of high caspofungin (Merck, Rahway, NJ, USA) levels, termed paradoxical growth was tested over a range of concentrations varying from 0.03 to 256 µg ml^{-1} and MICs were determined using prominent inhibition as an endpoint corresponding to 50% (MIC_{50}) [33, 218]. The paradoxical effect was defined as a progressive increase in cell growth occurring at least two drug dilutions above the MIC, following 48 hours incubation [218].

Data Analysis

The SI mean values displayed by the different isolates after CFW staining were compared using the Student's *t*-test. Significant effects were accepted at $p<0.05$. The SPSS Statistics 17.0 Software for Windows was used to perform the statistical analysis. All experiments were performed in triplicate.

Results and Discussion

The cytometric protocol was optimized using four *C. albicans* strains with known differences in chitin contents: *chs3*Δ*/chs3*Δ, *pga31*Δ/Δ, *pga62*Δ/Δ and the reference strain SC5314. These strains are deleted in genes that are involved in chitin synthesis (*chs3*Δ*/chs3*Δ) or encode GPI-proteins that are involved in cell wall biosynthesis or in cell wall salvage pathways (*pga31*Δ/Δ and *pga62*Δ/Δ) [128, 216]. A range of CFW concentrations was tested and 2.5 µg CFW ml^{-1} revealed to be the concentration to achieve the best resolution to differentiate the chitin content of the four strains used as controls. The reference strain

SC5314 had significantly higher SI values ($p<0.001$) than strains *chs3*Δ*/chs3*Δ and *pga31*Δ/Δ and had lower values when compared to the *pga62*Δ/Δ strain (figure 1). Flow cytometry chitin measurements were concordant with the chitin levels determined by the quantification of glucosamine released by acid hydrolysis, previously obtained by others [128, 215, 216]. Caspofungin treatment of the reference strain SC5314 led to a significant increase in chitin content ($p<0.001$), contrasting with the mutant strains where caspofungin did not produce any effect (figure 1). The chitin levels obtained after caspofungin exposure of the reference and *chs3*Δ/Δ strains are in agreement with those achieved by the classic method performed by other authors [128].

Furthermore, results obtained by flow cytometry were consistent with epifluorescence microscopy observations (data not shown). The flow cytometric protocol for chitin quantification is considerably less laborious and more accurate in comparison with the previously described methods since a large amount of cells (50000) are randomly evaluated, without operator interference. Given the variation in yeast morphology such as cell shape and size, different species may emit different levels of autofluorescence, which arises from endogenous fluorophores. This autofluorescence emission analyzed under epifluorescence microscopy or flow cytometry results in a background "noise" which may interfere with the quantification of fluorescence emitted by stained cells [217]. To avoid autofluorescence interference, especially when fluorescence emitted by cells from different species is compared, normalization of data is mandatory [217]. This was achieved through the calculation of a SI which provides sensitivity and reliability to the output data and enables comparison of the fluorescence emitted by cells with distinct morphologies. With this approach we can expect a possible normalization in intra and inter laboratory results.

The relationship between the CFW staining index and the caspofungin susceptibility phenotype displayed by clinical strains is detailed in Table 1 and figure 2. Among the distinct species included in this study, *C. parapsilosis*, *C. tropicalis* and *C. albicans* clinical isolates showed a higher CFW staining index, and therefore a higher cell wall chitin content

comparing to *C. glabrata* and *C. krusei* (figure 2). *C. neoformans* showed intermediary levels (figure 2).

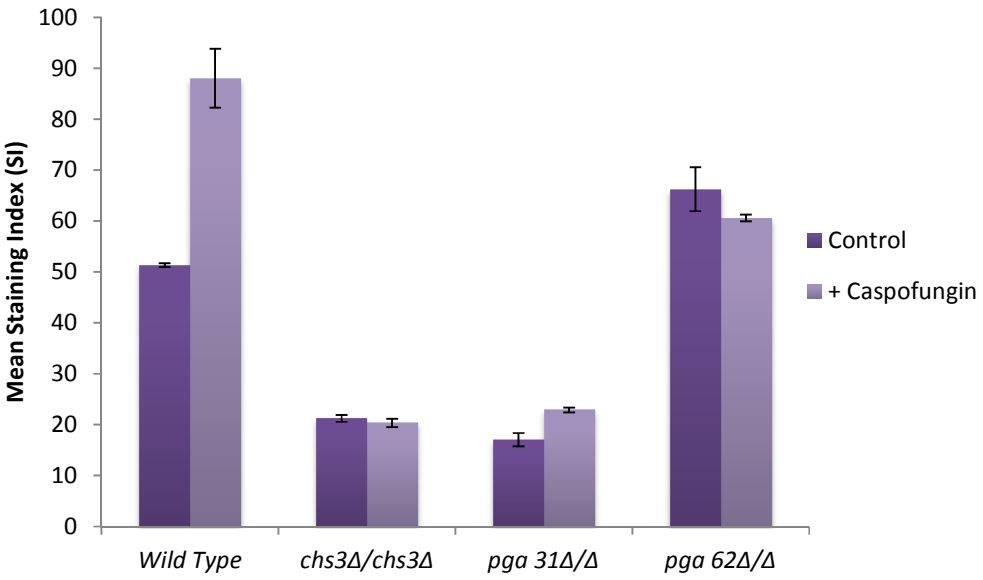


Figure 1. Cell wall chitin content of reference and *chs3*Δ*/chs3*Δ, *pga62*Δ/Δ and *pga31*Δ/Δ strains. A suspension of 10^6 cells ml^{-1} was stained with 2.5 µg CFW ml^{-1} and the intensity of fluorescence was quantified by flow cytometry. The SI mean values displayed by the different strains were determined after three independent experiments. Reference and mutant strains had significantly differences ($p<0.001$) in SI mean values, revealing diverse chitin levels in the cell wall. After caspofungin exposure, only reference strains showed a significant increase in chitin levels ($p<0.001$).

Table 1. *In vitro* antifungal susceptibility and paradoxical effect of caspofungin (CFS) against *Candida* spp and *Cryptococcus neoformans* clinical isolates. Minimal inhibitory concentrations (MIC; μg ml^{-1}) were determined using prominent inhibition as an end point, corresponding to 50% (MIC_{50}), according to CLSI protocol.

Yeast	Strain code	Source	CFS MIC_{50} (μg ml^{-1}) / Phenotype	Paradoxical growth (mean values) Start point/ end point (μg ml^{-1})
C. glabrata	Cg1	Blood	>32/ NS	NF
	Cg2	Blood	0.125/ S	NF
	Cg3	Peritoneal fluid	32/ NS	NF
	Cg4	Fecal	0.125/ S	NF
	Cg5	Peritoneal fluid	0.5/ S	NF
	Cg6	Blood	0.25/ S	NF
C. parapsilosis	Cp1	Peritoneal fluid	4/ NS	NF
	Cp2	Blood	2/ S	NF
	Cp3	Blood	4/ NS	NF
	Cp4	Blood	4/ NS	16/64
	Cp5	Blood	0.5/ S	NF
C. tropicalis	Ct1	Blood	0.5/ S	8/16
	Ct2	Peritoneal fluid	0.5/ S	NF
	Ct3	Pus	4/ NS	16
	Ct4	Pus	4/ NS	16
C. krusei	Ck1	Urine	1/ S	NF
	Ck2	Blood	1/ S	NF
	Ck3	Bronchial secretions	1/ S	NF
	Ck4	Bronchial secretions	1/ S	NF
C. albicans	Ca1	Blood	0.5/ S	16/32
	Ca2	Blood	0.5/ S	16/32
	Ca3	Blood	0.5/ S	16/32
C. neoformans	Cn1	Blood	16/ NS	NF
	Cn2	Blood	16/ NS	NF
	Cn3	Blood	16/ NS	NF
	Cn4	Blood	32/ NS	NF

NS non susceptible phenotype; S susceptible phenotype; NF not found

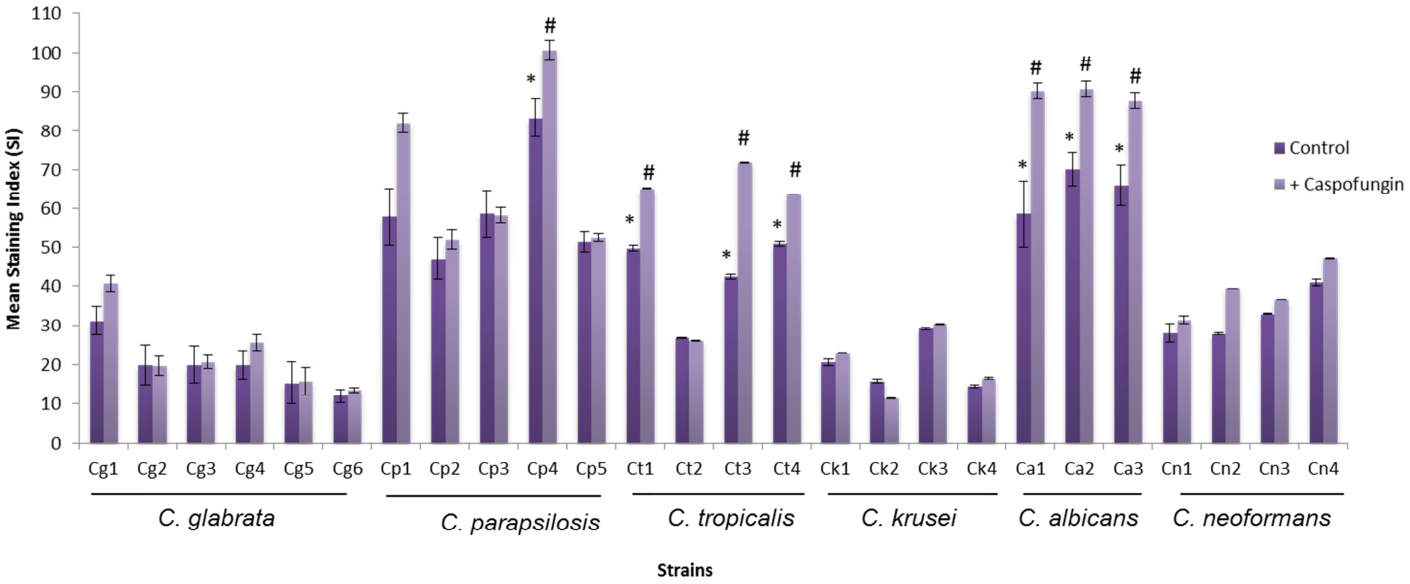


Figure 2. Cell wall chitin content of *Candida* spp and *Cryptococcus neoformans* clinical isolates in the absence and presence of caspofungin. Yeast cells were stained with 2.5 µg CFW ml^{-1} and fluorescence emitted was quantified by flow cytometry. Higher staining index (SI) values were observed in strains that showed a paradoxical growth (*) in the presence of caspofungin. # shows significantly different chitin levels after caspofungin treatment.

Notably, several *C. parapsilosis* and *C. tropicalis* (Cp4, Ct1, Ct3 and Ct4) isolates showed a significant increase in chitin level ($p<0.001$) in comparison with other isolates from the same species (figure 2). Interestingly, these strains, along with all *C. albicans* isolates tested, exhibited a paradoxical growth in the presence of high caspofungin concentrations. The ability to grow at high caspofungin concentrations has been frequently described among *C. albicans*, *C. parapsilosis* and *C. tropicalis* [218] and has been suggested to relate to a compensatory increase in cell wall chitin [128, 130]. This salvage mechanism strengths cell wall damaged by exposure to echinocandins. Chitin quantification by flow cytometry revealed to be a highly sensitive method. It enabled the detection of different chitin levels, which allowed us to validate the association between a higher amount of cell wall chitin and paradoxical growth in the presence of caspofungin concentrations well above the MIC. Also, when yeast cells were treated with caspofungin for two hours, a significant increase in

chitin levels were obtained, especially in strains that showed the paradoxical effect (Cp4, Ct1, Ct3, Ct4, Ca1, Ca2 and Ca3) (figure 2). The finding that caspofungin treatment stimulates chitin biosynthesis has also been described by other authors [130, 216, 218]. The clinical relevance of this *in vitro* effect is yet uncertain. Although unrelated to resistance, the paradoxical effect may represent a drug tolerance mechanism and an adaptive response to the presence of caspofungin. In contrast, *C. glabrata*, *C. krusei* and *Cryptococcus neoformans* strains showed an absence of paradoxical growth, displaying the lowest chitin levels. Interestingly, the accuracy of this novel methodology can predict the occurrence of this paradoxical effect within a few minutes, representing a valuable tool for detection of an antifungal compensatory mechanism in the presence of high antifungal concentrations, namely echinocandins.

The flow cytometry protocol described herein may constitute a useful tool to evaluate the inhibition of chitin synthesis by new drugs presently under development.

Part III

*FKS*2 mutations associated with decreased echinocandin susceptibility of *Candida glabrata* following anidulafungin therapy

Background

Fungal pathogens have become progressively more responsible for human disease. Although *C. albicans* is still the most frequent agent of fungaemia in U.S.A., the incidence of candidosis by non-*albicans* species is increasing, with *C. glabrata* being the second causing agent of fungaemia in U.S.A. and in some European countries [6, 48]. *C. glabrata* infections represent a serious concern due to the inherent high mortality rate, to patient comorbidities as well as to the predisposition to rapidly develop azoles resistance [7, 52, 59]. As such, there has been an urge to develop new therapeutic alternatives. A new class of antifungal agents has arisen, including caspofungin, micafungin and more recently anidulafungin, which inhibit the β (1, 3)-glucan synthase. This enzyme is responsible for producing a key component of the fungal cell wall and is encoded by the *FKS* genes and includes a regulatory subunit Rho-1p.

Due to their excellent clinical effectiveness and safety, these antifungals became the first-line therapy in many hospitals for the treatment of invasive candidosis [10-12]. To date, case reports describing primary or secondary echinocandin resistance in candidosis have been rare, however only caspofungin has been widely used. Reduced susceptibility to echinocandins has been linked to mutations in hot spot regions of *FKS* genes [31]. Specifically, mutations in *FKS2* have been shown to be associated with echinocandin resistance in *C. glabrata* acquired during caspofungin treatment [18, 125, 219]. Aside from glucan synthesis, chitin synthesis plays a fundamental role in maintaining fungal cell integrity during growth and morphogenesis. Previous studies have demonstrated a

significant increase in chitin content after exposure to different concentrations of echinocandins [130, 150]. This suggests that increased amounts of chitin could be responsible for reduced activity of echinocandins resulting as an escape mechanism to these agents [128].

The aim of this study was to unveil the underlying resistance mechanisms developed during prolonged anidulafungin treatment.

Herein, and for the first time, we describe a case of echinocandin resistance in a patient with candidosis by *C. glabrata* following anidulafungin therapy. Interestingly, during the hospital course two of the *C. glabrata* isolates turned out to be non susceptible to echinocandins displaying different mechanisms of resistance, possibly responsible for the patient's unfavorable outcome.

Case Report

A 71-year-old female was admitted to the Intensive Care Unit (ICU) with a diagnosis of acute pancreatitis developed after a laparoscopic cholecystectomy. The patient's medical history included arterial hypertension and dyslipidemia. An exploratory laparoscopic surgery was performed at the second day of admission at the ICU. The surgical findings were peritoneal necrosis, necrosis of the ileum and jejunum and pancreatic necrosis. During her hospital stay the patient was administered several antibacterial drugs namely tazobactam, piperacillin, meropenem, tigecycline and vancomycin. She had no previous antifungal exposure. Her medical condition was further complicated by multiple *Candida* infections, including disseminated candidosis and catheter related infections. Figure 1 summarizes the *Candida* isolates, the antifungal therapy administered and subsequent clinical response. Starting on day 6, she received a 200 mg load followed by 100mg/day of anidulafungin for 27 days. Cultures of multiple sites including blood, exudates, central venous catheter (cvc) and urine were positive for *C. albicans* and *C. glabrata.* While *C. albicans* fungaemia cleared upon 24 hours of anidulafungin therapy, *C. glabrata* persisted. At day 17 the patient received noradrenaline support since no clinical improvement could be observed, and was

subsequently submitted to a second exploratory laparoscopic surgery at day 30. The procedure revealed extensive cytoesteatonecrosis. Amphotericin B was then initiated and two days later the patient developed multiorgan failure. The patient died 34 days after admission. No autopsy was performed.

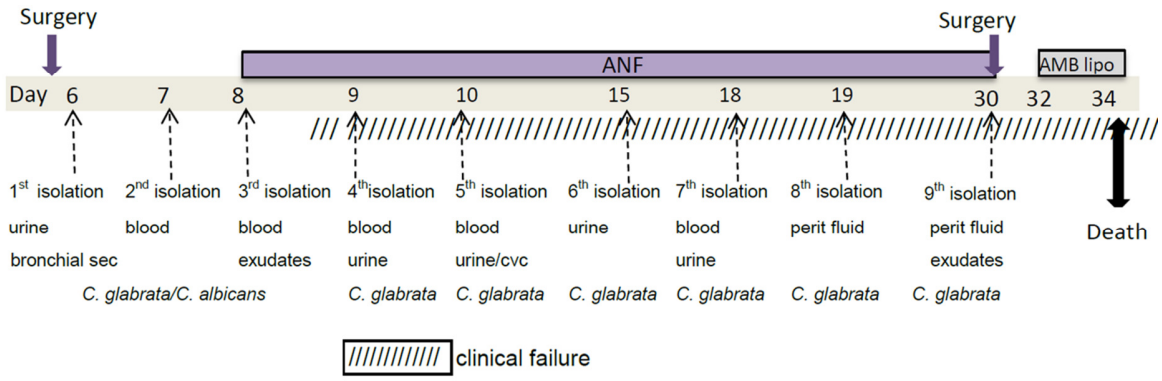


Figure 1. Antifungal therapy administered to the patient. Days after admission and fungal strains isolated from biological samples are also shown. AND, anidulafungin; AMB lipo, amphotericin B lipid complex.

Material and methods

Strains

The strains isolated from different body sites throughout the course of infection (figure 1 and table 2) were characterized by Vitek YBC identification cards (BioMérieux, Paris, France). For each laboratorial experiment, strains were subcultured twice on YPD agar (1% yeast extract, 2% peptone, 2% glucose, and 2% agar) medium for 48 hours at 35°C.

Microdilution susceptibility testing

Assessment of the antifungal susceptibility profile of all clinical isolates was performed according to the CLSI M27 A3 protocol in RPMI 1640 (Sigma) [33]. The minimal inhibitory concentration (MIC) of fluconazole (Pfizer, Groton, CT, USA), voriconazole (Pfizer), posaconazole (Shering-Plough, Kenilworth, NJ, USA), caspofungin (Merck, Rahway, NJ, USA),

anidulafungin (Pfizer), micafungin (Astellas Pharma US, Deerfield, IL) and amphotericin B (Bristol-Myers Squibb New York, USA) was determined [33]. Strains were classified as susceptible (S), susceptible-dose dependent (S-DD) or resistant (R) to azoles according to breakpoints defined by CLSI [33]. For caspofungin, anidulafungin and micafungin, MIC ≤2 μg/ml were considered S and those >2 μg/ml as non susceptible (NS) [33]. Although definitive breakpoints have not yet been established for amphotericin B and posaconazole, strains with MIC ≤1 μg/ml were considered susceptible [193].

Resistance stability

To assess resistance stability, the non susceptible isolates (7-1 and 9-2) were subcultured daily in the absence of anidulafungin for 30 days. Each strain was cultured in 10 mL drug-free RPMI 1640 at 35 °C, 150 rpm. The following day, aliquots were transferred into fresh medium. At each subculture, a 1-mL aliquot of the yeast suspension was mixed with 0.5 mL of 50% glycerol and frozen at −70 °C. Afterwards, the microdilution susceptibility testing of each collected strain to anidulafungin was repeated as detailed above.

Measurement of cell wall chitin content by flow cytometry

C. glabrata cells were grown in YPD broth medium at 35ºC under agitation until late log phase. A 10^6 yeast cells/ml suspension in distilled H_2O was stained with 2.5 μg/ml Calcofluor White (CFW) (Fluka), a specific chitin dye (excitation at 365 nm and emission at 430 nm), for 15 minutes at room temperature. Yeast cells were washed by centrifugation and the blue fluorescence (Pacific blue channel) was quantified in a flow cytometer BD FACSCANTO II (Becton Dickiinson, Madrid, Spain). The results were analyzed and processed with FACSDiva software. We determined the mean intensity of fluorescence emitted from yeast cell populations from three independent experiments.

Epifluorescence microscopy

In order to confirm flow cytometry assay results, *C. glabrata* cells were examined by epifluorescence microscopy. Yeast cells were grown and prepared as described for flow cytometry assays and stained with 25μg/ml CFW for 15 minutes. Upon staining, 30 μl of the cell suspension were placed on a slide and overlapped with vectashield fluorescence

mounting media (Vector Laboratories, Peterborough, UK). The stained suspensions were observed under an epifluorescence microscopy (400X) imager Z Apotome (Zeiss).

C. glabrata DNA extraction

For total DNA extraction, *C. glabrata* cells were grown overnight at 30ºC in 10 ml YPD broth at 180 rpm, collected by centrifugation and washed twice with sterile distilled water. Pellets were resuspended in 400 µl lysis buffer (20mM Tris, 1 mM EDTA, 100mM NaCl, 2% Triton X-100, 1% SDS) and 400 µl Phenol:Chloroform:Isoamyl alcohol 25:24:1; 0.3 g acid-washed glass beads were added and samples were strongly vortexed for 10 min with 1 min intervals on ice. Subsequently, 400 µl TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) were added and samples centrifuged at 14000 rpm, for 5min, at 4ºC. The aqueous phase was removed and an equivalent volume of Phenol: Chloroform: Isoamyl alcohol 25:24:1 was added. This phenol:chloroform extraction step was repeated twice. Genomic DNA was precipitated by 1ml of 100% ice-cold ethanol. Samples were centrifuged at 14000rpm for 20 min (4ºC), the supernatant was removed and the pellet washed twice with 70% ethanol. The pellet was allowed to dry, ressuspended in 200 µl of TE buffer and treated with 20 µg of RNase (Applichem, Darmstadt, Germany) by incubating at 37ºC up to 1h. For DNA final precipitation, 20 µl of 4M ammonium acetate pH 4.8 (Sigma –Aldrich, Germany), and 600 µl of ice-cold 100% ethanol (Applichem) were added and samples incubated overnight at -20ºC. Upon washing the pellet with ice-cold 70% ethanol, DNA was ressuspended in TE buffer to a final concentration of 2.0-2.5 µg/µl.

Random amplification of polymorphic DNA of all C. glabrata isolates

RAPD was performed according to methodology previously described by Bautista-Munoz et al. [220]. Primer OPE-18 (5' GGACTGCAGA 3') and OPA (5' AGCTGACCGT 3') at a final concentration of 0.4 µM were added to a PCR reaction mixture containing 1x Taq buffer, 10 ng of genomic DNA, 200 µM dNTPs, 1.5 mM MgCl2 and 1 U of Taq DNA polymerase (Fermentas). PCR was carried out in a Realplex Mastercycler instrument (Eppendorf) programmed as follows: an initial denaturation step of 2 min at 95°C followed by 38 cycles of 1 minute at 95°C, 1 minute at 36ºC, 2 minutes at 72ºC and a final extension step of 10

minutes at 72ºC. The RAPD patterns were obtained through electrophoresis in 2% agarose gel.

HS1 sequencing of FKS1 and FKS2 genes

Specific primers (table 1) were used for PCR amplification of HS1 of *FKS*1 and *FKS*2 genes. PCRs were performed in a Realplex Mastercycler instrument (Eppendorf) according to the amplification conditions: an initial denaturation step of 2 minutes at 96ºC followed by 30 cycles of 30 seconds at 96ºC, 30 seconds at 69.6ºC, 30 seconds at 72ºC and a final extension step of 10 minutes at 72ºC. PCR products were treated with ExoSAP-IT (USB Corporation OH, USA) and used as template for the sequencing reactions, performed with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The DNA products were purified with Sephadex G-50 Fine (GE Healthcare, UK) and sequenced in an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). The results were analyzed with the Sequencing Analysis 5.2 software from Applied Biosystems.

Table 1. Primers used for *C. glabrata FKS1* and *FKS2* HS1 amplification and sequencing.

Primers	Sequence (5'-3)	Reference
FKS1 1HS1F2	**CTTATGTTTGATTTTTGCA**	This study
FKS1 1HS1R	CCTTCAATTTCAGATGGAACTTGATG	[30]
FKS2-HS1F	**GTGCTCAACATTTATCTCGTAGG**	[219]
FKS2-HS1R	CAGAATAGTGTGGAGTCAAGACG	[219]

Data Analysis

Fluorescence mean values displayed by the different strains after CFW staining were compared using Student's *t*-test. Significance was accepted at $p<0.05$. The SPSS Statistics 17.0 Software for Windows was used to perform the statistical analysis.

The coding sequences of the *Candida glabrata FKS1* and *FKS2* genes (GenBank accession: XM_446406 and XM_448401) were aligned with those obtained from the clinical isolates. Alignments were analyzed in BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Results

Antifungal susceptibility profile

Twenty one isolates were recovered from the patient's blood and other biological samples throughout anidulafungin treatment (table 2). The majority of such isolates corresponded to *C. glabrata* (table 2). All strains were susceptible to echinocandins, except two strains one recovered from the blood (7-1) and the other from the peritoneal fluid (9-2), the 7th and 9th isolates, respectively (table 2 and figure 1). Regarding azoles and amphotericin B no resistance could be observed (table 2).

After subculturing the NS strains (7-1 and 9-2) in drug free RPMI medium during 30 days, the MIC values for both strains remained 4µg/ml (non-reverting strains 7-1NR and 9-2NR), confirming the stability of the non susceptible phenotype.

Table 2. *In vitro* antifungal susceptibility of successive *Candida* isolates to amphotericin B (AmB), fluconazole (FLC), voriconazole (VRC), posaconazole (PSC), caspofungin (CAS), anidulafungin (AND) and micafungin (MCA), determined accordingly to the CLSI protocol. Minimal inhibitory concentrations (MIC; µg/ml) were determined using prominent inhibition, corresponding to 50% (MIC_{50}) or 100% (MIC_{100}), as an end point.

Strain number	Days after admission	Isolate	Sample	MIC_{100} AmB	MIC_{50} FLC	MIC_{50} VRC	MIC_{50} PSC	MIC_{50} CAS	MIC_{50} AND	MIC_{50} MCA
1-1	6	*C. glabrata*	urine	0.5	8	0.5	1	0.06	≤0.06	≤0.06
1-2	6	*C. albicans*	bronchial secretion	1	32	1	1	0.125	≤0.06	≤0.06
2-1	7	*C. glabrata*	blood	0.125	16	2	2	0.125	≤0.06	≤0.06
2-2	7	*C. albicans*	blood	0.5	0.25	≤0.015	0.06	0.06	≤0.06	≤0.06
3-1	8	*C. albicans*	blood	0.5	0.25	≤0.015	0.06	0.06	≤0.06	≤0.06
3-2	8	*C. glabrata*	blood	0.25	16	2	0.5	0.125	≤0.06	≤0.06
3-3	8	*C. albicans*	exudate	≤0.06	≤0.125	≤0.015	≤0.03	≤0.06	≤0.06	≤0.06
3-4	8	*C. albicans*	exudate	0,25	0,25	≤0,015	≤0,03	≤0,06	≤0,06	≤0.06
3-5	8	*C. glabrata*	exudate	0.5	8	0.5	2	≤0.06	≤0.06	≤0.06
3-6	8	*C. glabrata*	exudate	0.125	16	2	2	0.125	≤0.06	≤0.06
4-1	9	*C. glabrata*	blood	0.25	4	2	1	0.125	≤0.06	≤0.06
4-2	9	*C. glabrata*	urine	1	32	1	1	0,125	≤0.06	≤0.06
5-1	10	*C. glabrata*	blood	0.25	1	2	1	0.125	≤0.06	≤0.06
5-2	10	*C. glabrata*	urine	0.125	16	2	2	0.125	≤0.06	≤0.06
5-3	10	*C. glabrata*	cvc	0.5	8	0.5	1	0.06	≤0.06	≤0.06
6-1	15	*C. glabrata*	urine	0.25	8	0.5	1	0.06	≤0.06	≤0.06
7-1	18	*C. glabrata*	blood	0.25	8	0.5	1	>32	4	4
7-2	18	*C. glabrata*	urine	1	4	0.25	0.125	0,25	0,25	≤0.06
8-1	19	*C. glabrata*	peritoneal fluid	0.5	8	0.5	1	0.06	≤0.06	≤0.06
9-1	30	*C. glabrata*	exudate	0.5	8	2	1	0.125	≤0.06	≤0.06
9-2	30	*C. glabrata*	peritoneal fluid	0.5	8	2	1	32	4	8

Measurement of chitin content

To evaluate whether anidulafungin (AND) resistance was related to an increase in chitin cell wall content, the two susceptible (5-1 and 8-1), two non susceptible strains (7-1 and 9-2) and the two non-reverting strains (7-1NR and 9-2NR) were compared regarding their chitin content.

Flow cytometry was used to measure fungal chitin through assessment of fluorescence emitted by Calcofluor White (CFW). CFW is a fluorescent dye that binds specifically to fungal chitin and the resulting fluorescence intensity is considered proportional to chitin cell wall content [209].

When compared to the other strains, only the non susceptible strain 7-1 displayed a significant increase ($p<0.0051$) in the fluorescence mean value after CFW staining, indicative of a higher chitin content (figure 2). This result is concurrent with epifluorescence microscopy observations, showing strain 7-1 a higher staining intensity, with no abnormalities in the distribution of chitin (blue fluorescence), compared to strain 9-2 (figure 2a and b, respectively).

Regarding the non-reverting strains (7-1NR and 9-2NR), after subculturing for 30 days in the absence of antifungal the chitin content decreased when compared to strains 7-1 and 9-2. Strain 7-1NR showed a significant lower chitin content than strain 7-1 (p=0.0106) (figure 2).

Genetic similarity between the different isolates recovered from the patient

Random amplification of polymorphic DNA (RAPD) was carried out in isolates from different biological samples to determine their isogenicity. Using primer OPE-18 and OPA-18 [220] an identical band profile was displayed by all isolates with exception of strain 9-2 recovered from the peritoneal fluid (figure 3).

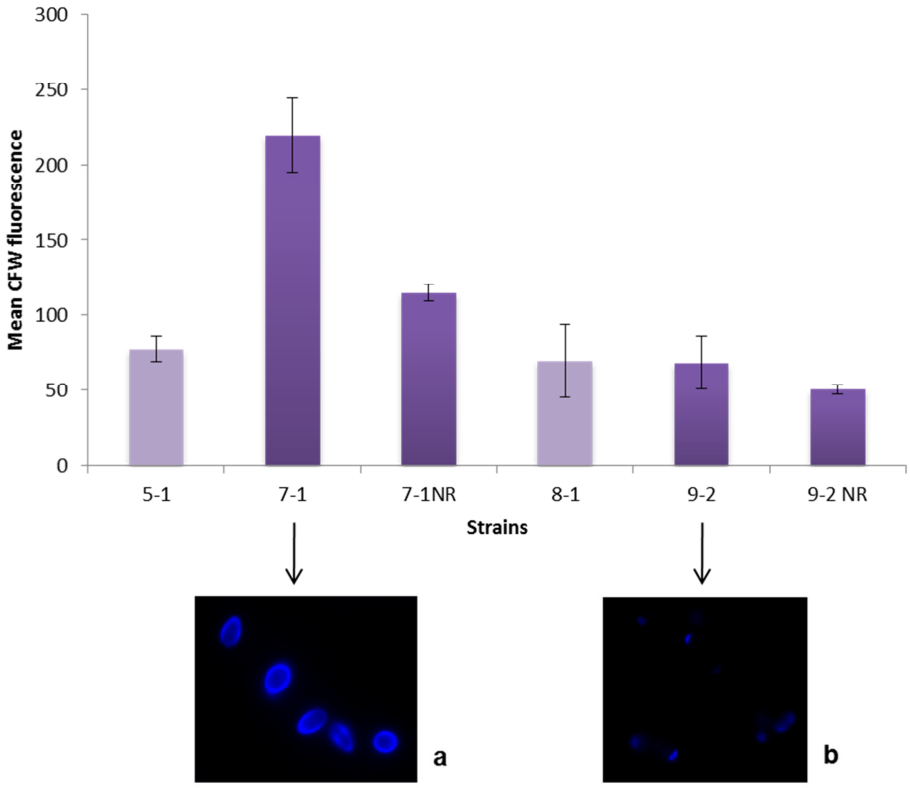


Figure 2. Relative cell wall chitin content from susceptible (light grey) and non susceptible (dark grey) strains. A suspension of 10^6cells/ml was stained with 2.5 µg/ml Calcofluor White (CFW) and the intensity of fluorescence was quantified by flow cytometry. The mean CFW fluorescence was determined from three independent assays.

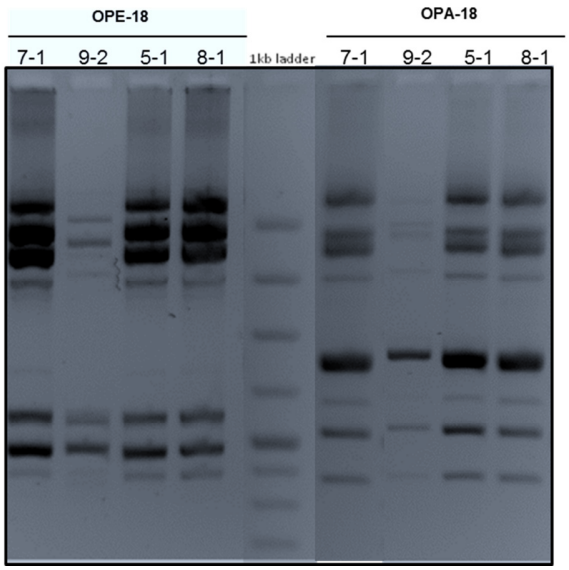


Figure 3. Random amplification of polymorphic DNA gel patterns of *C. glabrata* isolates 7-1, 9-2, 5-1 and 8-1 obtained with primers OPE-18 and OPA-18.

FKS1 and FKS2 sequence analysis

To determine if anidulafungin resistance was associated with mutations in the target genes, genomic DNA extraction of *C. glabrata* isolates was performed and the hot spot 1 (HS1) regions of *FKS1* and *FKS2* genes were amplified and sequenced. No sequence alterations were observed in HS1 of the *FKS*1 gene, however, several point mutations were found in HS1 of the *FKS*2 gene, most of them corresponding to synonymous substitutions that did not result in amino acid changes (table 3).

Furthermore, isolate 9-2, which was recovered following a long period of antifungal treatment, displayed a C-T mutation at position 1987. This mutation leads to a substitution of serine 663 by proline in HS1 of *FKS2* (table 3). In the same HS, a deletion of 3 nucleotides was found in the blood isolate 7-1, which results in the deletion of one of the two consecutive phenylalanines in positions 658 and 659 (table 3). These results are consistent with previous reports, describing increased resistance to echinocandins associated with the mutations S663P and Phe659 deletion in the *FKS2* gene [125].

Table 3. Mutations in HS1 of the *FKS2* gene from *C. glabrata* clinical isolates. All point mutations correspond to synonymous substitutions, except for the T to C substitution in position 1987 which led to a replacement of serine 663 by proline. The CTT deletion (1977-1979) results in the deletion of phenylalanine 659.

SNP	A1956G	C1959T	1977-9 CTT deletion	T1987C	T2046C	T2119C	C2191T
Codon	AAA-AAG	TAC-TAT	TTCTTG-TT…G	TCT-CCT	TAT-TAC	TTG-CTG	CTA-TTA
AA	Lys	Tyr	ΔPhe659	Ser663Pro	Tyr	Leu	Leu
***C. glabrata* isolates**	All	All	7-1	9-2	All	All	All

SNP – single nucleotide polymorphisms; AA - aminoacid

Discussion

Echinocandins are considered to be advantageous over other existing antifungals, mainly azoles, due its clinical effectiveness and safety profile [25, 221]. While the three echinocandins, caspofungin, micafungin and anidulafungin share the same target, *in vivo* potency, mechanism of resistance, and spectrum, discrepancies *in vitro* have been observed [125]. In the absence of serum, caspofungin seems to be less potent than the two others drugs, however, such differences are minimized in the presence of 50% of serum and were confirmed in *in vivo* models [125, 126, 222]. We report for the first time the *in vivo* acquisition of echinocandin resistance following anidulafungin therapy in a patient with *C. glabrata* invasive candidosis. Our study describes anidulafungin resistance acquisition associated to an increase in caspofungin and micafungin MIC, suggesting the development of cross-resistance between these three echinocandins. Although MIC values are usually considered predictors of clinical responses to antimicrobial therapy in invasive fungal infections, no strong correlation has actually been found between *in vitro* susceptibility test results and clinical outcome [7, 223-226]. It is considered that high *in vitro* MIC values for echinocandin drugs are correlated to clinical failure, however some reports describing high MIC values have not been shown to be good predictors of treatment outcome [227, 228]. The authors justified it as the majority of the MIC values found were close to breakpoint (2µg/ml) while the sporadic clinical failures were related to only strains displaying high MIC values (>2µg/ml) [31].

Our clinical case however depicts a correlation between *in vitro* results and *in vivo* efficiency of the drug. MIC values were well above the breakpoint in the blood (7-1) and peritoneal fluid isolates (9-2) from a patient under anidulafungin therapy who developed clinical failure. After RAPD analysis, *C. glabrata* isolates were shown to be isogenic, including the non susceptible blood strain (7-1), except the last non susceptible *C. glabrata* isolate, obtained from the patient's peritoneal fluid (9-2) prior to her death. Considering surgical procedures, the evidence of CVC infection and the long ICU stay (34 days), where

nosocomial infections are likely to occur, it is plausible that this strain had an exogenous source.

The acquisition of resistance to echinocandins in several *Candida* species has been associated with amino acid substitutions in two highly conserved regions of Fks1p or Fks2p [31]. Deletion of both alleles of the *FKS1* gene is lethal in *Candida* spp. and point mutations in *FKS1* and *FKS2* lead to reduced caspofungin susceptibility [11, 31, 125]. The highest frequency of resistance-associated mutations is found within hot spot 1 [229]. HS1 is a highly conserved region among the Fks family, hence amino acid changes in this region implicate a modification in the presumed echinocandin target and thus a reduced susceptibility phenotype. However, not all nonsynonymous mutations confer a similar resistance phenotype. A weaker resistance phenotype (caspofungin MIC≤2µg/ml) has been described for mutations occurring in the C-terminal of the HS1, whereas substitutions in the Ser645 lead to a stronger phenotype, even in a heterozygous form [11]. In our study, we identified this sort of mutation (C to T) leading to the Ser663Pro substitution in *FKS*2 HS1 of the non susceptible strain isolated from the peritoneal fluid.

Within the same region, a deletion of 3 nucleotides encoding Phe in position 659 was detected in the NS isolate 7-1 from blood. This deletion has previously been described as responsible for echinocandin resistance [125]. Furthermore, this isolate displayed an increase in cell wall chitin content compared to that of the other isogenic strains. The significant increase in chitin content following *in vitro* echinocandins exposure has been demonstrated by several investigators and suggested to be an escape or salvage pathway mechanism to echinocandins [128, 130, 150]. Up-regulation of the cell wall integrity pathway in *C. albicans* upon cell wall damage increases chitin content and has been correlated to paradoxical attenuation of caspofungin activity at clinically relevant supra-MIC concentrations [130]. Although this effect has not been observed in *C. glabrata*, some studies have reported the incomplete killing of some isolates due to the increase of *SLT2* and *CHS3* expression [14, 150, 218, 230]. Chitin and β1, 3-glucan represents key structural polysaccharides in fungal cell walls responsible for cell shape and structural rigidity [202]. Mutations in glucan synthase genes that reduce glucan levels in cell wall stimulate a salvage

pathway in which chitin synthesis is increased to restore the strength of the wall matrix [204]. Previous clinical case reports did not assess the cell wall chitin content of the isolates as a possible mechanism of resistance. In our study, a flow cytometry method was used to quantify the cell wall chitin. To unveil whether resistance of the blood isolate 7-1 was due to the Phe659 deletion or to the high chitin amount, daily subcultures in drug free medium were performed during 30 days. The NS phenotype remained, however the chitin content decreased. Attending to such results we can conclude that resistance of strain 7-1 was a consequence of the deletion found in *FKS*2 gene. Nevertheless, the initial high chitin level in strain 7-1 might indicate a form of drug tolerance and an adaptive response to the presence of anidulafungin *in vivo*. Whilst the Ser663Pro substitution in *FKS*2 HS1 seems to be exclusively responsible for the NS phenotype of the peritoneal fluid isolate. The chitin content found in this isolate was similar to that found on susceptible strains (9-2).

Overall, our findings suggest that structural alterations in the HS1 of *FKS*2 molecule due to the Ser663Pro substitution and a Phe659 deletion lead to a dramatic decrease in echinocandin efficiency. Although just a few cases of echinocandin resistance development have been so far reported, our case report emphasizes the crucial need of antifungal susceptibility surveillance in patients under extended echinocandin therapy.

Part IV

An Alternative Respiratory Pathway on *Candida krusei*: Implications on Susceptibility Profile and oxidative stress response

Background

Over the past few decades authors have documented increases in the rate of candidaemia by non- *albicans* species of *Candida*, such as *C. krusei*, especially in critically ill and immunocompromised patients with hematologic malignancies [52].

Fluconazole is often used in the prophylaxis and treatment of candidaemia and is the first-line therapy for this condition [10]. Among non-*albicans* species, *C. krusei* is the only that is predictably fluconazole resistant [231]. Antifungal drug tolerance can also be modulated by metabolic adaptability mechanisms, including alterations in the respiratory mitochondrial pathway [161, 162] . This fact had been previously assessed in *C. glabrata*, *C. albicans* and *C. parapsilosis* regarding its influence on fluconazole and caspofungin [161-163].

In eukaryotic organisms the energy necessary for growth, development, reproduction and stress response is acquired through the ATP, synthesized during the mitochondrial respiration, where cytochrome c oxidase acts as a terminal oxidase in the reception of electrons and converting oxygen into water. Within the mitochondrial respiratory chain, another route mediated by the alternative oxidase (AOX) (a mitochondrial enzyme), can be found in plants, in certain protozoa and fungi [232-236]. This AOX is insensitive to cytochrome pathway inhibitors, such as antimicin A or cyanide, but is specifically inhibited by salicylhydroxamic acid (SHAM) and confers a cyanide-resistant respiration through an alternative respiratory pathway (ARP) to such organisms [233]. The AOX is located on the matrix side of the inner mitochondrial membrane and plays an important role in susceptibility to azole antifungals in *C. albicans* [163]. The alternative respiratory chain can

be activated by stress situations like the presence of antifungals or oxidative inductors, thus leading to drug tolerance and to the reduction of generation of intracellular reactive oxygen species (ROS) [237].

In an attempt to explain the contribution of mitochondrial respiration in the intrinsic fluconazole resistance displayed by *C. krusei*, we assessed the existence of an alternative respiratory pathway and its influence upon fluconazole resistance and tolerance to oxidative stress.

Material and Methods

Drugs and chemicals

Fluconazole was obtained from Pfizer (Groton, CT, USA) and stock solutions were prepared according CLSI M27 A3 protocol and maintained at -70 ºC until use [238]. Stock solutions of salicylhydroxamic acid (SHAM; 200 mM; Sigma-Aldrich, Germany) and potassium cyanide (KCN; 1 M; Sigma) were prepared in DMSO (Sigma) and distilled water, respectively. Hydrogen peroxide (H_2O_2; 30% v/v) was obtained from Merck. Plumbagin and menadione were obtained from Sigma and stock solutions (100 mM) were prepared in 95% ethanol. Dihydrorhodamine 123 (DHR123) was obtained from Molecular Probes (Molecular Probes, Eugene, OR, USA).

Strains and culture conditions

A total of 25 *Candida krusei* (9 from respiratory secretions, 1 from blood, 11 vaginal, 3 faecal and the type strain ATCC 6258 from the American Type Culture Collection), previously identified using VITEK II system (BioMérieux, Paris, France), all resistant to fluconazole were used in this study. *Candida albicans* SC5314 strain was used as positive control for the presence of AOX specific protein. The strains were grown in Sabouraud dextrose broth (Difco) at 30 ºC, in an orbital shaker at 150 rpm until late exponential growth phase ($O.D._{640}$ 1.5). *Debaromyces hansenii* IGC2968 (LGC Standards S.L.U., Barcelona, Spain) was used as the positive control for the presence of an alternative respiratory pathway, assessed by

oxygen consumption. *Saccharomyces cerevisiae* BY4742 (EUROSCARF, Frankfurt, Germany) was used throughout the study as the negative control for the presence of the alternative oxidase assessed by oxygen consumption, immunoblotting and ROS production. These control strains were grown in a mineral medium with vitamins and 2% (w/v) glucose until stationary growth phase ($O.D._{640}$ 2.5 and 3, respectively) [232].

Alternative respiratory pathway

Late exponential phase cultures were centrifuged (18 000g) for 4 minutes at 4 ºC and washed twice with cold sterile water. An amount of 1.5 g (wet weight) pellet was ressuspended in 100 ml of phosphate-buffered 50 mM, pH 6.0 with 0.1% cicloheximide (Sigma, St. Louis, MO, USA) and 6 ml of suspension were incubated in a small reactor at 28 ºC. The O_2 consumption was continuously measured with a Clark type electrode YSI model 5775 (YSI Incorporated, Yellow Springs, Ohio, USA) after the addition of 200 µl of glucose 1.55 M (Difco Laboratories, Detroit, MI, USA), 3.2 mM of KCN and/or 3.2 mM of SHAM (Sigma-Aldrich, Germany). The presence of an alternative oxidase (AOX) was considered whenever the oxygen consumption pattern was resistant to KCN but sensitive to SHAM.

Chequerboard microdilution assay

Checkerboard assays were performed with all *C. krusei* strains, in the presence of fluconazole and SHAM, using the protocol described in the Clinical Microbiology Procedures Handbook [239]. The concentration range used was 0.125 µg fluconazole ml^{-1} to 64 µg fluconazole ml^{-1} and 0.6 to 20 mM for SHAM. The minimal inhibitory concentrations (MIC) of each compound were determined according to CLSI M27 A3 and S3 protocol in RPMI 1640 (Sigma) [238]. Fractional inhibitory concentration of fluconazole (FIC_A) was calculated as the MIC of fluconazole in combination/MIC of fluconazole alone and FIC_B is the MIC of SHAM in combination/MIC of SHAM alone. The fractional inhibitory index (FIX) was calculated as follows: $FIX= FIC_A + FIC_B$. The interpretation of FIX was as recommended: ≤0.5, synergistic effect; >0.5 to <4.0, no interaction; ≥4.0, antagonistic effect [239].

Assessment of intracellular ROS accumulation

Intracellular ROS accumulation was estimated in order to study the influence of an AOX upon *C. krusei* oxidative stress tolerance. After being washed and ressuspended in PBS, 10^7cells ml^{-1} were treated with ROS inductors such as H_2O_2 (0.4 mM), menadione (0.5 mM) and plumbagin (0.003 mM) for 15, 30 and 60 minutes, at 35 ºC under 150 rpm. Yeast cells were also treated with fluconazole and voriconazole at MIC concentrations for 1 hour. All the treatments were repeated following the pre-incubation of yeast cells with 3.2 mM of SHAM, an inhibitor of the AOX, during 30 minutes. Control cells were treated with a DMSO concentration similar to that used in cell samples pre-treated with SHAM. The cells were then collected by centrifugation and ressuspended in PBS. Free intracellular ROS were detected with 15 µg dihydrorhodamine 123 ml^{-1} (Molecular Probes, Eugene, OR, USA) [240, 241]. Cells were incubated during 90 min at 30 ºC in the dark, washed in PBS and 5 µl were placed in a glass slide and overlapped with vectashield fluorescence mounting media (Vector Laboratories, Peterborough, UK). The stained suspensions were visualized under epifluorescence microscopy (40X) (Olympus BX61). In each condition, a minimum of 500 cells from 3 different replicates were counted and the percentage of stained cells (cells displaying ROS) was determined.

Chronological life span

Overnight cultures of *C. krusei* strains were incubated in YPD broth until stationary phase (day 0 of chronological life span) [240]. Cultures were treated without (control) and with 3.2mM SHAM, 0.25mM and 0.5mM of menadione alone and in combination with 3.2mM of SHAM. Survival was assessed daily by counting colony-forming units (CFUs) from cultures aliquots in YPD agar plates beginning at day 0 (when viability was considered to be 100%).

Preparation of Mitochondrial extracts

Mitochondria extracts were isolated from *S. cerevisiae*, *Candida albicans* SC5314 and *C. krusei*. Cells were grown to stationary phase, harvested and cell wall was digested with zymolyase buffer [2 M Sorbitol-D, Phosphate buffer 1 M (pH 7.5), zymolyase 20.000 U, 125 mM β-mercaptoethanol, 0.5 M EDTA] at 30ºC for 1 hour. Protoplasts were disrupted with

lysis buffer [Sorbitol-D 0.5 M, Tris 20 mM, EDTA 1 mM, and 2.85 mM phenylmethanesulphonyl fluoride (PMSF)] using a Potter homogenizator. Mitochondria extracts were separated, washed by high speed centrifugation at 12000 r.p.m. for 15 min at 4ºC (Beckman Coulter, JA-25.50 Rotor) and ressuspended in sorbitol buffer [0.5 Sorbitol-D, 5 mM EDTA, 50 mM Tris]. Protein concentration was determined by Bradford method [242] and protein aliquots of mitochondrial extracts (40 µg) were stored at -20ºC.

Immunoblotting

Mitochondrial protein extracts were resolved on a 12% SDS gel and transferred to a nitrocellulose membrane. The membrane was blocked with phosphate-buffered saline (TBS) with 0.1% Tween 20 (TBST) containing 5% skim milk, followed by incubation with antibodies against AOX proteins (1:100) of *Sauromatum guttatum* (Agrisera AB, Vännas, Sweden) in TBST containing 1% skim milk. After washing with TBS, the membranes were incubated with the respective secondary antibody, HRP-conjugated anti-mouse IgG at a dilution of 1:5000 and detected by enhanced chemiluminescence.

Statistical analysis

All experiments were performed in triplicate. Mean values were compared using Student`s T-test whenever indicated. A p value <0.05 was considered statistically significant.

Results

Presence of a cyanide resistant respiration pathway in C. krusei

In order to investigate whether an alternative respiratory pathway (ARP) was present in clinical isolates of *C. krusei*, measurements of O_2 consumption were performed in the presence of 3.2 mM of cyanide, an inhibitor of the classical respiratory chain (by inhibiting the cytochrome c oxidase complex), and SHAM, an inhibitor of the ARP (by inhibiting the alternative oxidase, AOX). In *C. krusei* strains, O_2 consumption stopped only after the addition of SHAM (figure 1a, representative example). This observation was made for all the 24 *C. krusei* clinical isolates, with ATCC 6258 type strain and with the positive AOX control

strain *Debaromyces hansenii* 2968 (figure 1b). In *Saccharomyces cerevisiae* BY4742, the negative AOX control strain, the oxygen consumption stopped soon after the addition of cyanide (figure 1c). The results indicate that *C. krusei* cells have a cyanide resistant respiration pathway promoted by the presence of an AOX.

AOX expression

Mitochondrial preparations were immunoblotted with a monoclonal antibody raised against *S. guttatum* AOX, but also recognize *C. albicans* AOX [243]. In all *C. krusei* clinical isolates mitochondria AOX was clearly detectable as well as in *C. albicans* SC5314 strain (figure 2). Mitochondria isolated from the negative control strain, *S. cerevisiae* BY4742, showed no reactivity with AOX antiserum (figure 2).

Fluconazole-SHAM combination did not influence fluconazole activity

In order to study the influence of the alternative respiratory pathway upon fluconazole resistance by *C. krusei*, we explored the *in vitro* combination of fluconazole and SHAM using the chequerboard methodology. SHAM produced no impairment of antifungal activity even at a concentration of 10mM. The addition of SHAM did not change MIC values for fluconazole, resulting in an indifferent effect in all *C. krusei* isolates.

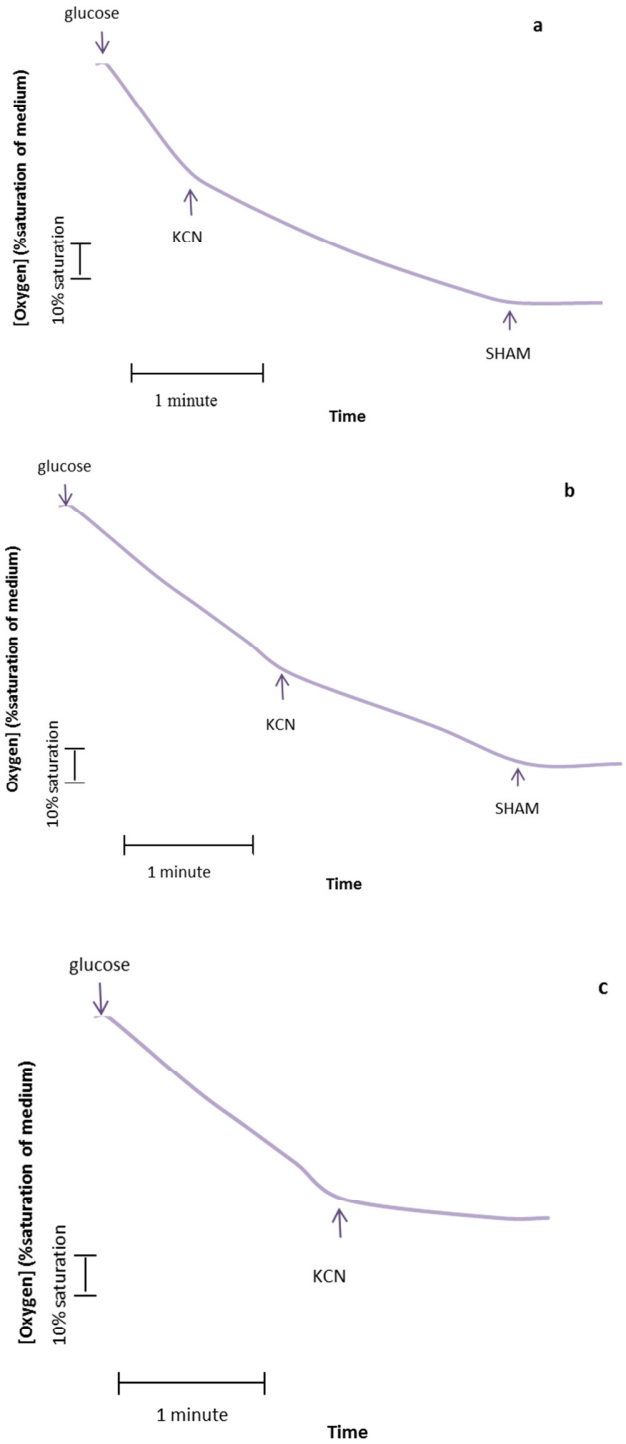


Figure 1. Effect of KCN and SHAM upon oxygen consumption by *Candida krusei* clinical strain (representative example) **(a)**, *Debaryomyces hansenii* (alternative oxidase positive control strain) **(b)** and *Saccharomyces cerevisiae* (alternative oxidase negative control strain) **(c)**. Oxygen consumption was measured with an oxygen electrode at 28 ºC. Where indicated (arrows), glucose (0.051 mM), KCN (3.2 mM) or SHAM (3.2 mM) were added.

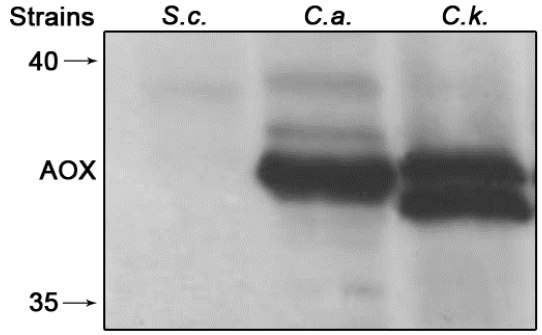


Figure 2. Representative example of the presence of an AOX in *Candida krusei*. Immunoblot analysis of AOX levels in 40 µg of mitochondrial extracts of *Saccharomyces cerevisiae* (*S.c.*), *Candida albicans* SC5314 (*C.a.*) and *Candida krusei* (*C.k.*).

The presence of an alternative respiratory pathway in C. krusei relates to reduced ROS accumulation

To assess the influence of the alternative respiratory pathway upon oxidative stress response we measured intracellular ROS accumulation with and without SHAM, using DHR123. This fluorochrome enters the yeast cell as a freely permeable dye, which is converted to rhodamine 123 and subsequently localized in the mitochondria. The conversion from the non-fluorescent to the fluorescent molecule is entirely dependent upon the presence of oxidation products. After treatment with the ROS-inducing agents, H_2O_2, plumbagin, menadione and with azoles, the percentage of stained cells (cells with ROS accumulation) was calculated and compared with values displayed by non treated cells, in the presence and absence of SHAM (3.2 mM). No significant differences regarding ROS accumulation were observed with the AOX negative strain, *S. cerevisiae* BY4742, in the presence or in the absence of SHAM (figure 3). In contrast, after treatment with ROS inducing agents, *C. krusei* cells treated with SHAM produced significantly ($p< 0.001$) more endogenous ROS than the cells with the unblocked alternative respiratory pathway (without SHAM) (figure 3). Significant differences in ROS production ($p= 0.008$) were also accomplish with fluconazole (figure 3). All *C. krusei* tested strains displayed similar results.

Chronological life span decreased in *C. krusei* strains incubated with menadione when compared to untreated cultures (figure 4). The viability of *C. krusei* strains reduced significantly after 3 days of incubating the cultures with menadione plus SHAM (figure 4).

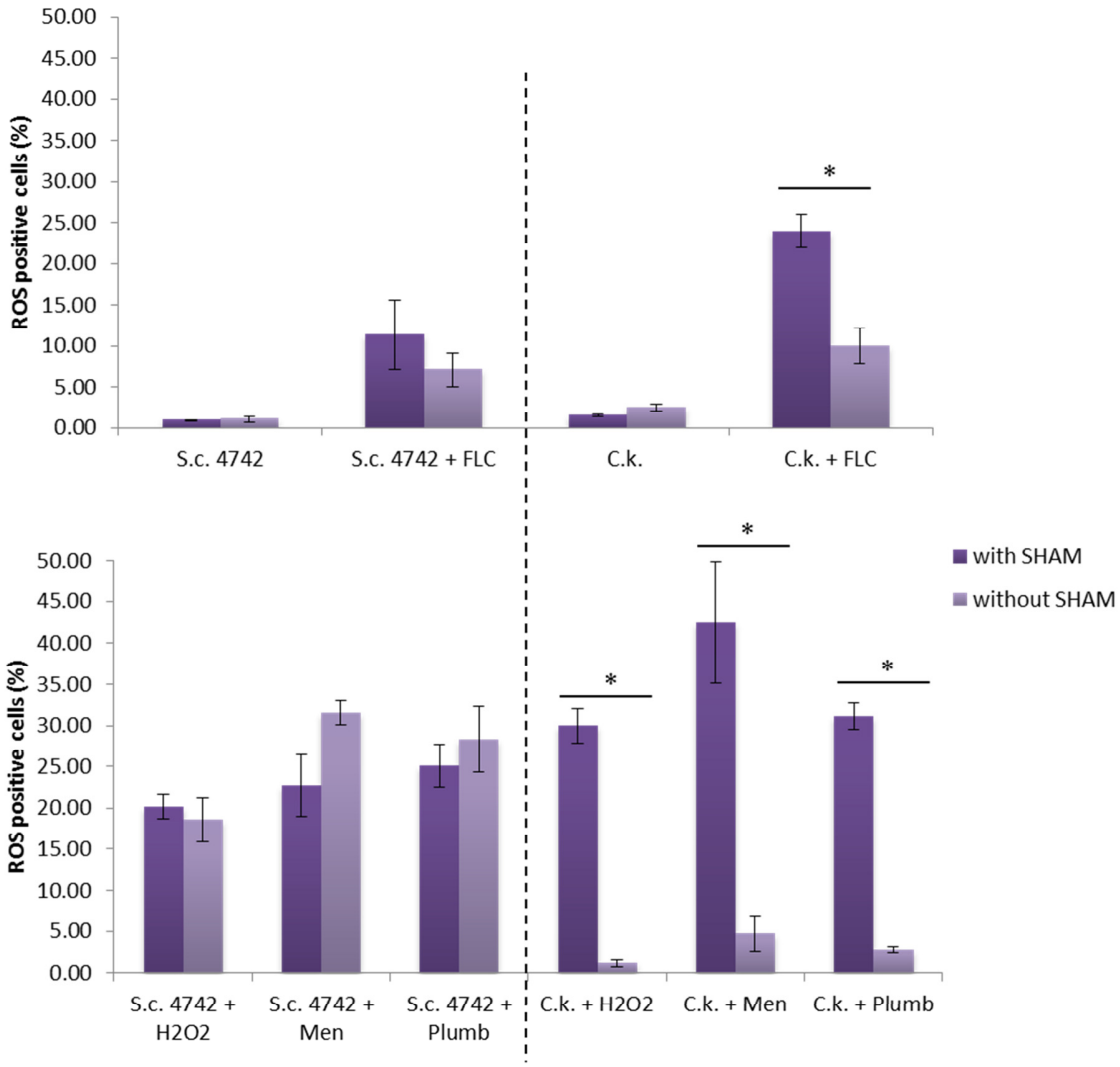


Figure 3. Effect of fluconazole (FLC) and oxidative inductors (menadione – Men; plumbagin – Plumb; hydrogen peroxide – H_2O_2) upon intracellular ROS accumulation by a clinical *Candida krusei* (C.k.) strain (representative example) and a negative control strain *Saccharomyces cerevisiae* (S.c.), with or without the addition of SHAM. ROS accumulation was calculated and expressed as the percentage of DHR123 stained cells (*p<0.05).

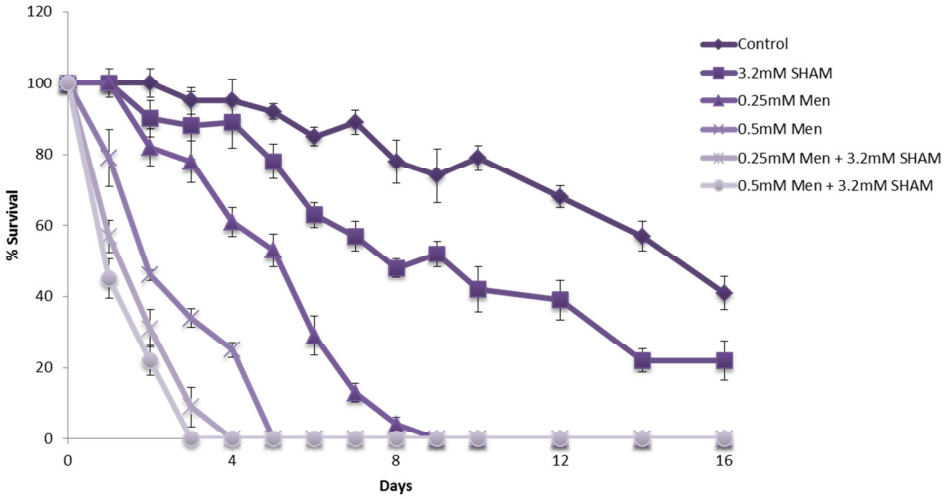


Figure 4. Chronological life span of a *C. krusei* strain. Strains were incubated without (control) and with 3.2mM SHAM, 0.25mM and 0.5mM of menadione (Men) alone and in combination with 3.2mM of SHAM. Survival was assessed daily by counting colony-forming units (CFUs) from cultures aliquots in YPD agar plates beginning at day 0 (when viability was considered to be 100%).

Discussion

Several authors have stressed the relevance of the mitochondrial respiration and its influence upon metabolic behavior, stress environment adaptability and antifungal drug tolerance [161-163, 244] . A mitochondrial alternative respiratory pathway (cyanine-resistant) occurs in all higher plants, in many fungi and in some protozoa [245, 246]. Such a pathway uses electrons from the ubiquinol pool to reduce oxygen to water, bypassing the complex III and the cytochrome oxidase complex, two sites of energy conservation in the main respiratory chain. An alternative oxidase (AOX), sensitive to salicylhydroxamic acid (SHAM) and resistant to cyanide, is responsible for this alternative pathway [247]. The cyanide-resistant respiration has been previously described in *C. albicans* and *C. parapsilosis* [162, 163, 234]. In this study we described for the first time the presence of an alternative respiratory pathway mediated by an alternative oxidase in *C. krusei*.

The elucidation of the oxidative stress responses in yeast has considerable clinical interest, as it is involved in invasion and colonization of host tissues by yeast pathogens as well as during the defensive mechanisms triggered by phagocytes. All aerobic organisms inevitably generate a range of ROS, including superoxide anion, hydrogen peroxide and hydroxyl radical during oxygen metabolism. If not quickly and effectively eliminated from the cells, ROS will trigger a large number of oxidative reactions in cellular systems that possibly lead to cell death [248]. In the course of an *in vivo* infection, the formation of ROS and other oxidants radicals by phagocytes play a crucial role in the intracellular destruction of the pathogen [249]. ROS attack almost all essential cell components, including DNA, proteins and lipids [250]. In a recent study, *C. krusei* appears to be resistant to ROS and to possess a potent antioxidant system enabling deep systemic infections [251]. We decided to evaluate the difference regarding oxidative stress response before and after the blockade of the alternative respiratory pathway. It has been previously described that in *C. albicans*, fluconazole is able to induce the production and accumulation of ROS [252]. In our study, we showed that fluconazole induced a low percentage of ROS formation by *C. krusei* cells. These results may suggest that the fungistatic mechanism of this azole is not based upon ROS formation. However, when the AOX was inhibited by SHAM, an increase in the intracellular ROS levels was evident. Attending to these facts, we can conclude that AOX activity allows the yeast cells to reduce ROS accumulation when challenged by antifungals like fluconazole, leading to drug tolerance, like in *C. albicans* [163]. According to several authors, the AOX has a metabolic and antioxidant role and its presence may be considered a potential virulence attribute of pathogenic fungi [163, 253, 254]. The importance of AOX activity upon resistance to oxidative stress was evident when the oxidative stress inductors, H_2O_2, menadione and plumbagin were assayed. After treatment with such compounds, ROS accumulation was low. However, the scenario changed significantly when the AOX activity was blocked by SHAM. To confirm our hypothesis that the presence of an alternative respiratory pathway could protect *C. krusei* from oxidative stress, we assessed ROS accumulation in the presence of fluconazole and the other oxidative stress inductors by the negative control strain, *S. cerevisiae* (AOX-). The results obtained regarding ROS

accumulation, in the presence or absence of SHAM, were not significantly different. When testing *C. albicans* AOX mutant strains, Yan and co-workers also obtained no significant differences in the amount of ROS generation [163]. Regarding the chronological life span assays we could conclude that the decreased viability of *C. krusei* strains in the presence of menadione and with the AOX blocked is associated with an increased level of cell ROS.

Our results showed clearly that the inhibition of the expression of the AOX was associated with intracellular ROS accumulation, unveiling its effect of the alternative respiratory pathway on oxidative damage. Although fluconazole resistance was unrelated to the presence of the ARP, we can consider that it confers antifungal tolerance, which may give yeast cells enough time to develop long-term genetically stable resistance mechanisms [22].

The alternative respiratory pathway is a potential target that should be taken into account considering the development of new therapeutic strategies in the case of *C. krusei* infections. Attending to its selective effect, SHAM should be used in combination with azoles, in order to reduce resistance due to oxidative stress and consequently the virulence of *C. krusei*.

Part V

Propofol lipidic infusion promotes resistance to antifungals by reducing drug input into the fungal cell

Background

Discrepancies between *in vivo* and *in vitro* susceptibility to antifungals discourage microbiologists and clinicians regarding the routine use of susceptibility testing methods. Although *in vitro* resistance usually correlates with clinical resistance, high susceptibility *in vitro* is not always related to clinical success. Ultimately, the mortality rates are unacceptably high in patients treated with antifungals that showed high *in vitro* efficacy [255].

Propofol is an intravenous hypnotic agent very popular for induction and maintenance of general and intravenous anaesthesia. It is commonly administered in Intensive Care Units to critically ill patients, often under mechanical ventilation, which represent a high risk group for health care related infections. The use of propofol has been previously associated to an increased risk for infection, although some controversy still remains [256, 257]. It was proposed a low risk of contamination whenever providing standard hygienic precautions [256, 258].

Nevertheless, other observations described the lipid emulsion of propofol as a good culture medium to support the growth of *Candida albicans* and *Escherichia coli* [259, 260]. Additionally, other reports associated post-surgical infections with the extrinsically contamination of propofol infusion [258, 261]. Propofol has also been shown to inhibit a variety of functions of neutrophils *in vitro*, although such effect was not so evident *in vivo* [262].

We have studied the effect of the infusion of propofol and its lipidic vehicle upon antifungal susceptibility of *Candida*. A promotion of resistance due to a decreased input of the antifungal drugs was found.

Materials and Methods

Strains

Twenty clinical strains of *Candida* spp. (5 *C. albicans*, 5 *C. tropicalis*, 5 *C. glabrata* and 5 *C. parapsilosis*) were studied. *C. albicans* 95-190, resistant to azoles by overexpression of efflux pumps genes (CDR1 and CDR2), was used during cytometric approach (strain kindly gift by Prof. Theodore White). Until testing, yeasts and moulds were kept frozen in Brain-Heart broth (Difco Laboratories, Detroit, MI, USA) with 5% glycerol. For each experiment, the strains were subcultured twice on Sabouraud agar (Difco) at 35ºC, 48 hours for *Candida*.

Drugs and Chemicals

Propofol infusion Fresenius® (Kabi, France) at stock concentration of 1% was used. Propofol vehicle (soya bean oil, egg lecithin, glycerol, sodium hydroxide and sterile water) was also assayed. Fluconazole and voriconazole were obtained from Pfizer (Groton, CT, USA), amphotericin B from Bristol-Myers Squibb (New York, USA), itraconazole from Janssen-Cilag (Beerse, Belgium) and posaconazole from Shering-Plough (Kenilworth, NJ, USA). Antifungals drugs were maintained in stock solution at -70ºC until use. [^{3}H]-labelled itraconazole was supplied by Janssen-Cilag. Sodium azide was purchased from Sigma (Sigma-Aldrich, Germany).

Growth assays

After cultivation of *Candida* strains in Sabouraud agar medium (Difco, Detroit, MI, USA), a $5x10^{6}.ml^{-1}$ blastoconidia suspension of *Candida* was prepared in phosphate buffer saline (PBS) (Sigma) and 100µl were added in two parallel serial dilutions of propofol infusion (stock solution at 1%) and its vehicle (both at 0, 1.25, 2.5 and 5 $mg.ml^{-1}$ final concentrations) in RPMI 1640 culture medium (Sigma), PBS and plain propofol infusion in a final volume of

500µl. RPMI is a hydrophilic medium, however, solubility problems were not found. For *Candida* strains, samples were collected after 3 hours incubation at 37ºC, the cells were observed under phase contrast microscopy (Leitz Larborlux K) and the percentage of budding and germ tube formation for *C. albicans* were determined [263].

Susceptibility testing

For *Candida* spp., the minimal inhibitory concentration (MIC) to fluconazole, voriconazole, posaconazole and amphotericin B (tested concentration range: 0.125-64 $\mu g.ml^{-1}$, 0.03-16 $\mu g.ml^{-1}$, 0.03-16 $\mu g.ml^{-1}$ and 0.03-16 $\mu g.ml^{-1}$, respectively) were determined accordingly the CLSI protocols M27-A2 (formerly NCCLS) [33]. Strains were classified as susceptible (S), susceptible-dose dependent (S-DD) and resistant (R) to fluconazole according to breakpoints defined by CLSI [33]. For voriconazole MICs ≤1 $\mu g.ml^{-1}$ were considered S, MIC =2 $\mu g.ml^{-1}$ considered S-DD and MIC ≥4 $\mu g.ml^{-1}$ considered R [264]. Although susceptibility breakpoints have not yet been established for amphotericin B and posaconazole, strains with MIC ≤1 $\mu g.ml^{-1}$ were considered susceptible [193, 265]. Minimal fungicidal concentration (MFC) to all antifungals was also determined. The content of each well containing drug concentrations to and higher than the MIC, and also the positive growth control were transferred to Sabouraud dextrose agar plates and incubated at 35°C for 48h, as previously described [266]. The MFC was the lowest drug concentration that killed ≥99% of the final inoculum.

The susceptibility tests to the antifungals mentioned above were repeated in the presence of the propofol infusion or its vehicle in three distinct concentrations (1.25, 2.5 and 5 $mg.ml^{-1}$). Since propofol infusion and its vehicle are opaque solutions, making impossible MIC determination, the content of each well containing antifungal + propofol drugs was cultured for MFC determination and values compared with the MFC to antifungals alone.

Flow cytometry analysis

Yeast cells were incubated at 150 rpm, overnight, until late exponential growth, in Sabouraud broth (Difco) at 37ºC. Yeasts cells were harvested after centrifugation and a $1x10^6$ $cells.ml^{-1}$ suspension was prepared in PBS supplemented with 2% glucose (GH

solution) and later divided into aliquots of 1 ml. The cells were then incubated with different concentrations of propofol infusion (0, 1.25, 2.5 and 5 $mg.ml^{-1}$) at 37ºC for 90 minutes and afterwards washed thrice, ressuspended in sterile water supplemented with 2% glucose and stained with 0.5 µM FUN1 (Molecular Probes, Europe BV, Leiden, Holland) for 30 minutes at 37ºC. A Beckman Coulter XL-MCL flow cytometer (Beckman-Coulter Corp., Hialeah, FL, USA) equipped with a 15 nm argon laser was used. From each suspension 30000-50000 cells were analysed. The intensity of fluorescence emitted by cells treated with propofol infusion was determined at FL2 (575 nm) and compared with non-treated cells (control). In parallel experiments, yeast cells were treated with 0.1 mM sodium azide during 30 minutes, prior to incubation with propofol, as previously described [145],, in order to block efflux pumps; thereafter, the same flow cytometry analytical protocol was used.

Intracellular accumulation of [^{3}H]-labelled itraconazole

Candida cells were initially incubated under similar conditions as previously described for flow cytometry assays. The cells were harvested by centrifugation at 5000 rpm for 10 minutes at 4ºC, washed thrice, ressuspended in PBS at a final concentration of $2.5x10^8$ $cells.ml^{-1}$ and incubated with 0, 1.25 and 5 $mg.ml^{-1}$ of propofol infusion at 37ºC, with continuous shaking at 300 rpm for 30 minutes [145]. Parallel experiments were prepared, but also involving a pre-incubation of the yeasts cells with sodium azide at 0.1 mM. The cells were washed thrice and [^{3}H]-labelled itraconazole was added to yeast suspensions at a final concentration of 3 µM, as previously described [145]; the cells were incubated in glass vials at 37ºC, with continuous shaking (300 rpm), during 1 hour and then harvested by centrifugation at 5000 rpm for 10 min at 4ºC, washed thrice with 3ml of ice-cold PBS containing 10 µM unlabelled itraconazole. The pellets were later ressuspended in 500 µl of PBS and the radioactivity was determined, following the addition of a scintillation cocktail (Optiphase “Hiphase3”, Perkin-Elmer), in a liquid scintillation counter (LKB Wallac, 1209 RackBeta).

Lipidic vehicle experiments

All the described experiments were repeated in the presence of the propofol lipidic vehicle used in Fresenius® formulation (soya bean oil, egg lecithin, glycerol, sodium hydroxide and sterile water).

Statistical analysis

The effects of different concentrations of propofol upon germination of fungal cells and MFC values of the distinct antifungals were compared using one–way analysis of variance (ANOVA) and Student's *t*-test. Significance was accepted at $p<0.05$. The SPSS 14.0 program for Windows was used to perform the statistical analysis. All susceptibility experiments were run in duplicate and growth and radioactivity assays in triplicate.

Results

In *Candida* strains, budding and germ tube formation were similar in presence of all tested concentrations of propofol infusion. In non-*albicans* strains, the incubation with 5µg.ml^{-1} of propofol infusion resulted in a significant increase of cells with buds when comparing to control (71.5% ± 7.46 *versus* 26.6% ± 4.15, *C. parapsilosis* $n=5$ as a representative example) ($p<0.001$). Conversely, a significant reduction of germ tube formation was observed in *C. albicans* strains comparing with non-treated yeasts (17.3% ± 6.29, *versus* 76.2% ± 8.69, $n=5$) ($p<0.001$).

The result of MIC determination revealed that all fungal strains were susceptible to the tested antifungals. Propofol infusion or its vehicle, at the tested concentrations, consistently promoted an increase of MFC mean values for *Candida* strains (Table 1), this effect being dose-dependent and statistically significant ($p<0.001$); such effect was invariably observed with all strains of *Candida* and with all antifungals, in some cases the mean values increasing over 4 fold. MFC values in the presence of 5mg.ml^{-1} of propofol infusion or its vehicle increased at least 2 dilutions in all strains (above the error rate of the method) for fluconazole and voriconazole, more than 3 dilutions for amphotericin B and 4 to 5 dilutions

for posaconazole and itraconazole. There was no large variability between the tested strains.

Flow cytometry analysis of *Candida* blastoconidia resulted in 98% of cells stained with FUN1, even after the incubation of sodium azide (figure 1b). However, after treatment with propofol infusion and stained with FUN1, a non-stained sub-population of cells, similar to autofluorescence, of around 15% was revealed soon after 90 minutes (figure 2c). This fact was unrelated with the incubation with sodium azide at a concentration able to block the efflux pumps (figure 1c). The azole resistant strain of *C. albicans* 95-190 (with overexpression of efflux pumps genes), after treatment with propofol infusion and stained with FUN1, revealed a non-stained sub-population, similar to other susceptible strains, even after the blockade of the efflux pumps with sodium azide. This effect was similar in presence of all the tested concentrations of propofol.

After 1 hour, the accumulation of [^{3}H]-labelled itraconazole was detected in blastoconidia cells (control cells) (figure 2). However, a decrease of 39% in intracellular [^{3}H]-labelled itraconazole was seen when yeast cells were incubated with propofol lipid infusion (figure 2). This effect was similar in presence of the different tested concentrations of propofol lipidic infusion and did not increase following the pre-incubation with sodium azide (figure 2).

The results obtained in the different assays were similar when performed with propofol lipidic vehicle.

Table 1. Minimal fungicidal concentration (MFC) values of *Candida* strains to AMB (amphotericin B), FLC (fluconazole), ITC (itraconazole), VRC (voriconazole) and PSC (posaconazole), determined by CLSI protocols, in the absence and presence of propofol infusion.

		Strains (n) MFC range µg.ml^{-1}			
Antifungals	Propofol (mg.ml^{-1})	*C. albicans* (5)	*C. tropicalis* (5)	*C. parapsilosis* (5)	*C. glabrata* (5)
AMB	0	0.25	0.25-1	0.06-0.125	0.25-0.5
	1.25	0.5-1	1	2	2
	2.5	0.5-2	1-2	2	2
	5	2-4	2	2-8	4-8
FLC	0	8-16	4-8	8-16	16
	1.25	16->64	>64	>64	>64
	2.5	16->64	>64	>64	>64
	5	16->64	>64	>64	>64
ITC	0	nd	nd	nd	nd
	1.25	nd	nd	nd	nd
	2.5	nd	nd	nd	nd
	5	nd	nd	nd	nd
VRC	0	0.06-0.25	0.25-0.5	0.5-1	4-8
	1.25	0.25->2	>2	>2	>8
	2.5	0.5->2	>2	>2	>8
	5	1->2	>2	>2	>8
PSC	0	0.06-0.125	0.03-0.125	0.06-0.125	1-2
	1.25	1-2	>2	2	2
	2.5	>2	>2	2	>2
	5	>2	>2	>2	>2

[nd] not done

* The mean value for each strain was considered

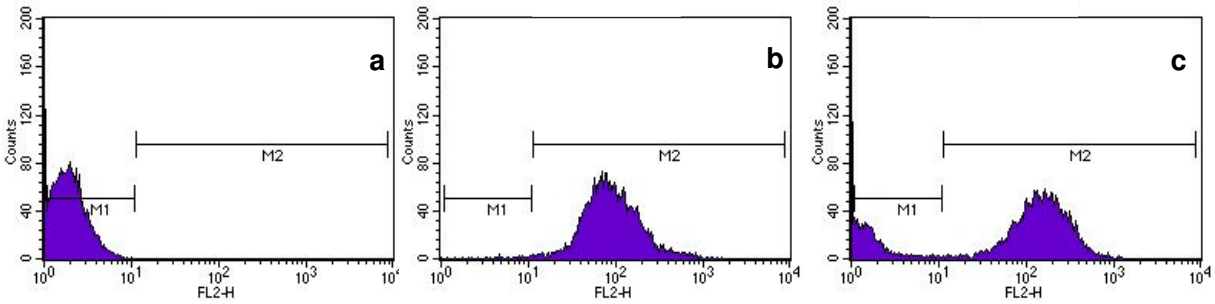


Figure 1. Flow cytometric histograms representing the emitted fluorescence after 90 minutes by: a. non-stained yeast cells (autofluorescence); b. cells treated with sodium azide and stained with FUN1; c. cells treated with sodium azide and 5 mg.ml^{-1} of propofol infusion and stained with FUN1 (strain of *Candida albicans* shown as a representative example).

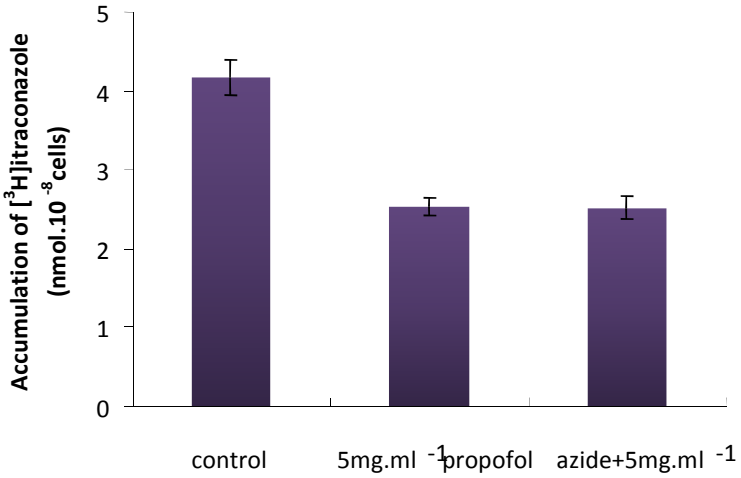


Figure 2. Effect of propofol up on [^{3}H]-labelled itraconazole accumulation in antifungal susceptible strain *C. albicans* ATCC 90028. The accumulation of itraconazole was measured in the absence and presence of 5 mg.ml^{-1} of propofol and after a prior incubation with 0.1mM sodium azide. Dispersion bars relates to standard deviation.

Discussion

Propofol administered in a lipid-based emulsion to patients has been shown to represent an excellent vehicle for supporting the growth of various microorganisms [259, 260]. In the present study we clearly showed that budding of *Candida* spp. was promoted when incubated in the presence of propofol infusion. It is important to emphasize that the propofol concentrations used in all assays are within the plasma levels often achieved in clinical practice [267, 268].

Due to the opacity of propofol infusion or its lipidic vehicle it was not possible to determine MIC values. As the conventional susceptibility phenotypes refer to MIC values, we could not establish the corresponding phenotypes after incubation with propofol. In the case of amphotericin B (a fungicidal to *Candida*) MFC, determined after culture in agar solid medium, usually corresponds to MIC values; for the other antifungals, MFC values are usually located within one or three dilutions above the MIC. Some *C. albicans* and *C. tropicalis* showed trailing phenotype (about 2 dilutions) for azoles. This common effect may influence the evaluation of MFC values. However, trailing is only azole dependent and was not affected by the presence of propofol infusion. Nevertheless, we could observe a significant promotion of the MFC value in presence of propofol infusion, in some cases up to 4 dilutions, suggesting that the strains became quite tolerant to the effect of all antifungals. FUN1 is a fluorescent probe that is converted by metabolically active yeasts in intracytoplasmic vacuolar structures [269]. We have used this probe to study susceptibility of yeasts after incubation during one to two-hours with the antifungals; a higher intensity of fluorescent in susceptible strains, decreasing in resistant strains was described [142, 144]. The decrease of the intensity of fluorescence could be explained by the presence of energy-dependent efflux pumps, which were reverted with sodium azide or several modulators [144, 269]. In the present study, the viable cells showed a decrease of the intensity of fluorescence in the presence of propofol which was not however reverted with sodium azide, even considering the resistant tested strain. Furthermore, cells were washed several times before the addition of the fluorescent probe in order to avoid its binding to propofol infusion. These results support that propofol infusion affected FUN1 access and/or

permeabilization, reducing the input of the fluorescent probe into the cell. We had also previously shown that [^{3}H]-labelled itraconazole accumulation could be a useful tool to confirm the existence of efflux pumps in resistant *Candida* strains to azoles [145]. The blockade of efflux pumps with sodium azide resulted in the reduction of input of [^{3}H]-labelled itraconazole in *Candida* cells, after incubation with propofol infusion. In fact, propofol infusion was responsible for the difficult access of itraconazole to the fungal cell.

Attending to the facts that i) the increase of MFC values was similar with all antifungal drugs, including the recently available posaconazole; ii) FUN1 could not stain a considerable percentage of the yeast cells in presence of propofol infusion and iii) a lesser amount of [^{3}H]-labelled itraconazole was present within such cells, even after the addition of sodium azide, we are forced to conclude that the promotion of antifungal resistance in presence of propofol lipidic infusion results from a reduced access and/or permeabilization of antifungal agents into the fungal cells. The same results were obtained when only the propofol vehicle was used, thus we had concluded that the major responsible for such effect was the lipidic nature of propofol infusion.

Several studies reported that the lipidic membrane of fungal cells plays an important role in susceptibility to azoles [270, 271]. Fluctuations in the lipidic environment affects, not only drug diffusion but also efflux pumps, coded specially by Cdr1 and Pdr5p, leading to multidrug azole resistance [272, 273]. In our study, as the efflux pumps were blocked with sodium azide, the increased MFC values probably resulted from poor diffusion of the antifungal drugs caused by the lipidic vehicle layer or drops deposited around the cells. The hypothesis that propofol could be binding the antifungal agent in culture medium reducing the free number of molecules available for penetrating the cells was also raised. MFC results were more evident when propofol was associated with itraconazole or posaconazole (increase of 4 to 5 dilutions), both lipophilic drugs. However, we must consider that different concentrations of propofol resulted in similar effects. The hypothesis that the lipidic vehicle of propofol might be playing a role in sterol homeostasis and changing the azole target, should be considered attending to other research [274]. Lipid uptake by the fungal cell was described in the presence of the azoles following longer incubation time

(overnight) [274]. Measuring sterols in azole-treated fungi in the presence or not of the lipidic vehicle of propofol could be interesting however, the incubation time with propofol on cytometric and radioactivity studies was very short to allow the incorporation of the lipid in membrane.

The assays described in this manuscript provided an opportunity to describe the effect of propofol infusion in antifungal drug resistance. We concluded that propofol infusion, due to its lipidic vehicle reduced the access and/or permeabilization to *Candida* cells to main antifungals administered to patients.

The described effect should raise the alert to a promoted risk of fungal infections in patients receiving propofol infusions, resulting from the fact that fungal strains become increasingly resistant to antifungals.

Part VI

Adrenaline stimulates efflux pumps activity, growth and mitochondrial respiration in *Candida albicans*

Background

Candida spp. are the most common agents of bloodstream fungal infections [6]. High morbidity and mortality rates are often observed, mainly among intensive care units (ICU) patients [7, 275].

Despite the *in vitro* susceptibility to antifungals, particularly to azoles, a poor clinical outcome is often reported [6, 7, 48, 276], thus suggesting the development of *in vivo* resistance. *Candida* may develop resistance mechanisms during medical therapy that ultimately may lead to clinical failure [51]. However, discrepancies between *in vitro* susceptibility and the patient outcome show that drug resistance is not the single factor for the clinical results. The progression of *Candida* infections is often observed despite aggressive antifungal treatment, especially among ICU patients [6, 7, 48]. Such patients are invariably submitted to multiple lifesaving medications. In a recent study of patients admitted in ICU with bloodstream *Candida* infections we have shown that those receiving vasoactive amines showed a significantly higher risk of fungaemia-related poor outcome [7].

In humans, catecholamines target adrenoceptors which are a class of G protein-coupled receptors (GPCRs). GPCRs are transmembrane spanning proteins that transduce an extracellular signal (catecholamine binding) into an intracellular event (G-protein activation). Although firstly detected in mammals it is now clear that these receptors followed closely the evolutionary spectrum from archaebacteria to humans. GPCRs are abundantly expressed in yeasts, showing an identical GTP binding site to human GPCRs [277-281].

Considering these homologies between yeasts and human cells, we hypothesized that adrenaline and other vasoactive amine administered to ICU patients might also act on

Candida. We aimed to clarify whether adrenaline, acting on *C. albicans* might promote antifungal tolerance, resistance or both of clinical isolates to azoles, amphotericin B and caspofngin. The results obtained underscore the role of non-antifungal drugs like catecholamines, often prescribed in ICU patients, as promoters of antifungal tolerance or resistance.

Material and Methods

Strains

The clinical isolates of *Candida albicans* (n=6) included in this study had been previously collected from blood cultures of patients with fungaemia admitted at different Intensive Care Units of Hospital São João, Porto, Portugal. All strains were susceptible to fluconazole, voriconazole, posaconazole, amphotericin B and caspofungin, according to the Clinical Laboratory Standards Institute (CLSI) M27-A3 protocol [282, 283]. *C. albicans* DSY 448 (*cdr1*Δ/Δ) [284], DSY 653 (*cdr2*Δ/Δ) [285], and DSY 654 (*cdr1*Δ/Δ *cdr2*Δ/Δ) [285] with selective deletion of efflux genes were used (kindly gifted by Prof. D Sanglard). *C. albicans* strains *gpr1* Δ / Δ (LDR8-5 strain) and *gpa2* Δ / Δ (NM8 strain) [277] (kindly provided by Prof. Patrick Van Dijck), Δ *ras1-2*/ Δ *ras1-3* (kindly provided by Prof. Gerald Fink) and *ste2* Δ / Δ, *ste3* Δ / Δ and *ste4* Δ/Δ [145, 279] (kindly provided by Prof. David Soll) were used and the respective wild-type strains SC5314, P37005, P57072. Until testing, all strains were kept frozen in yeast extract peptone dextrose agar (YPD) (Difco Laboratories, Detroit, MI, USA) with 40% glycerol. For each experiment, the strains were subcultured twice on YPD medium at 35°C for 48 hours and afterwards cultured in YPD broth at 35°C, under constant agitation.

Drugs and Chemicals

Fluconazole and voriconazole were obtained from Pfizer (Groton, CT, USA), posaconazole from Shering-Plough (Kenilworth, NJ, USA), amphotericin B from Bristol-Myers Squibb (New York, USA) and caspofungin from Merck (Rahway, NJ, USA). Antifungal drugs were prepared following CLSI recommendations [282, 283] and maintained in stock solutions at -70°C until use.

The adrenoceptor agonists adrenaline (mixed α_1, α_2, β_1 and β_2-adrenoceptor agonist) (Braun Medical, Barcarena, Portugal), noradrenaline (mixed α_1, α_2 and β_1-agonist) (Sigma-Aldrich, Germany), isoprenaline (selective β_1 and β_2-agonist) (Sigma), medetomidine (selective α_2-agonist) (Santa Cruz Biotechnology, Inc, Heidelberg, Germany) and phenylephrine (selective α_1-agonist) (Sigma) were used. Drugs were dissolved in distilled water and kept at -20ºC until use.

Sodium azide was purchased from Sigma and dissolved in distilled water. The fluorescent probes FUN-1 was acquired from Molecular Probes (Molecular Probes, Europe BV, Leiden, Holand) and Rhodamine 6G (Rh-6G) from Sigma.

Antifungal susceptibility testing of the clinical isolates

Antifungal susceptibility testing of all clinical isolates was performed according to CLSI M27 A3 protocol [282, 283] in RPMI 1640 (Sigma). The minimal inhibitory concentration (MIC) of fluconazole, voriconazole, posaconazole, caspofungin and amphotericin B was determined [282, 283]. Strains were classified as susceptible (S), susceptible-dose dependent (S-DD) and resistant (R) to azoles according to the breakpoints defined by CLSI [282, 283]. Although definitive breakpoints have not yet been established for amphotericin B and posaconazole, strains with MIC ≤1 μg/ml were considered susceptible [286]. For caspofungin a MIC ≤2 μg/ml was considered S and >2 μg/ml non susceptible (NS) [282, 283]. MICs of adrenaline and noradrenaline were also determined using the above mentioned CLSI protocol [282, 283] (tested concentration ranging from 5 to 545 μM).

The susceptibility tests with all the antifungals previously used were repeated in the presence of adrenaline (5, 27, 54, 108, 136, 273, 545 μM) and noradrenaline (11, 27, 136, 545 μM). Synergistic studies were performed according to the checkerboard procedure, as described in the Clinical Microbiology Procedures Handbook [239]. Fractional inhibitory concentration of drug A (FIC_A) was calculated as the ratio of the MIC of drug A in combination over the MIC of drug A alone. FIC_B was the ratio of the MIC of drug B in combination over the MIC of drug B alone. The fractional inhibitory index (FIX) was calculated as follows: $FIX = FIC_A + FIC_B$. The interpretation of FIX was as recommended: ≤0.5 synergistic effect; >0.5 to <4.0 indifferent effect; ≥4.0 antagonistic effect [239].

Efflux quantification by flow cytometry

a) Yeast cell culture

The yeast cells were incubated in YPD broth at 35ºC until mid-exponential growth phase (optical density at 600nm [OD_{600}]= 0.4) and harvested by centrifugation. A suspension with 10^6cells/ml was prepared in sterile distilled water supplemented with 2% glucose (Sigma).

b) FUN-1 and Rh-6G staining

Cells suspensions were incubated with 5, 27, 54, 108 µM of adrenaline for 90 minutes at 35ºC and stained with 0.5 µM of FUN-1 for 30 minutes, according to Pina-Vaz *et al* [145]. For Rh-6G staining, yeast suspensions were incubated with 5 µM Rh-6G for 30 minutes, washed twice and ressuspended in cold PBS (Sigma) supplemented with 10 mM of sodium azide. Yeast suspensions were kept in ice until cytometric analysis in a FACSCalibur flow cytometer (BD, Bioscience, Sydney). From each suspension 30,000 cells were analyzed and the intensity of fluorescence emitted by cells exposed to the different treatments was determined without staining (autofluorescence – af) and after FUN-1 and Rh-6G staining at FL2 (575 nm) and FL1 (525 nm), respectively. Results were compared with non-treated cells (control).

Studies with adrenoceptor agonists

Parameters like incubation time and agonist concentration were optimized using adrenaline. Adrenaline was tested at the concentrations of 5, 27, 54, 108, 136, 273 and 545 µM, for 30, 60 and 90 minutes.

To further characterize the adrenoceptor that mediates the effect of amines upon clinical isolates, yeast suspensions were also incubated for 90 minutes, at 37 ºC with noradrenaline (5, 27, 54, 108, 136, 273, 545 µM), isoprenaline (5, 27, 54, 108, 136, 273, 545 µM), phenylephrine (5, 27, 54, 108, 136, 273, 545 µM, 1, 3 mM) and medetomidine (0.01, 0.1, 1, 10 µM) [287].

Effect of adrenaline upon Rh-6G staining of Candida CDR1 and/or CDR2 mutant strains

Mutant strains DSY 448, DSY 653, DSY 654 and the wild-type strain were analyzed by flow cytometry following treatment increasing concentrations of adrenaline for 90 minutes and

staining with Rh-6G (according to the protocol described above). The amount of intracellular accumulation of Rh-6G was determined relatively to untreated cells (control) and compared between the different mutant strains.

Effect of adrenaline upon membrane receptor deleted strains

C. albicans selectively deleted in one of membrane receptors or proteins, *gpr1*Δ/ Δ, *gpa2*Δ/ Δ *[277]*, Δ *ras1-2*/ Δ *ras1-3* [276, 278], *ste2*Δ/ Δ, *ste3*Δ/ Δ and *ste4*Δ/ Δ [145, 279], and its respective wild-type strains, were incubated with 0, 5, 27, 54 and 108 µM of adrenaline for 90 min at 35ºC, stained with Rh-6G and analyzed according to the cytometric protocol described above.

Effect of adrenaline upon Candida CDR1 and CDR2 gene expression

C. albicans cell extracts for immunoblotting, previously exposed to 54 µM of adrenaline for 30, 60 and 90 minutes, were prepared by an alkaline extraction procedure as described previously [103]. Detection of Cdr1p and Cdr2p was performed as described previously [103]. Signals were revealed by exposure to Kodak BioMax MR films (GE Healthcare).

Effect of adrenaline upon C. albicans SC5314 strain growth

Following overnight culture in YPD broth medium a yeast suspension was diluted to an $O.D._{600}$ 0.07 in 50 ml of YEPD broth and incubated at 35ºC under agitation (150 rpm). Cells suspensions were exposed to the following conditions: no treatment (control), treatment with FLC 1 µg/ml (sub-MIC concentration), treatment with 54 and 108 µM of adrenaline, treatment with FLC 1 µg/ml plus 54 of adrenaline and treatment with FLC 1 µg/ml plus 108 µM of adrenaline. $O.D._{600}$ was registered repeatedly during 18 hours.

Effect of adrenaline upon oxygen consumption by C. albicans

Late log phase cultures of SC5314 strain were centrifuged (18000g) for 4 minutes at 4 ºC and washed twice with cold sterile water. An amount of 1.5 g (wet weight) pellet was ressuspended in 100 ml of phosphate-buffered 50 mM, pH 6.0 with 0.1% cicloheximide (Sigma, St. Louis, MO, USA) and 6 ml of such suspension were incubated in a small reactor at 28ºC and 200 µl of glucose 1.55 M (Difco Laboratories, Detroit, MI, USA) were added. The O_2 consumption was continuously measured during 90 minutes with a Clark type electrode

YSI model 5775 (YSI Incorporated, Yellow Springs, Ohio, USA) in the presence of 16 µg/ml FLC, with 0 and 54 µM of adrenaline, and the oxygen uptake rate was determined.

Data presentation

All susceptibility assays were performed at least thrice. Mean values were compared using Student`s T-test whenever indicated. A *p* value <0.05 was considered statistically significant. The determination of FUN-1 and Rh-6G staining, oxygen consumption and strain growth were made in triplicate and the respective standard deviations values determined. Potency values were expressed as pEC_{50} values, which are the negative logarithms of the molar concentration of the drug required to elicit half-maximal effect.

Results

Effect of adrenaline on antifungal susceptibility of C. albicans isolates – checkerboard results

Checkerboard assays were performed in order to evaluate the interactions between adrenaline and antifungal drugs. Adrenaline, as well as noradrenaline, did not show antifungal activity upon all isolates even at high concentrations (MIC values ≥ 545µM). However, the median FIX for fluconazole combined with adrenaline or noradrenaline was 5 (range 2-17) (antagonistic effect). Although an antagonistic effect was obtained, the MIC values did not reach a breakpoint, except for one clinical isolate (that changed to susceptible –dose dependent, MIC=16 µg/ml). In contrast to fluconazole neither the antifungal activity of amphotericin B nor caspofungin were changed by adrenaline.

Effect of adrenaline upon FUN-1 and Rh-6G staining

In order to elucidate the antagonistic effect of adrenaline on the antifungal action of fluconazole, flow cytometric evaluations were performed using FUN-1. FUN-1 is a fluorescent probe that is converted by metabolically active yeasts in intra-cytoplasm vacuolar structures [269]. This probe has been previously used to study susceptibility of yeasts to azoles [147, 213]. In susceptible strains a high intensity of fluorescence is detected, while in resistant strains fluorescence decreases [147, 213]. This decrease in fluorescence intensity is due to the activity of energy-dependent efflux pumps which

actively export FUN-1 to the extracellular environment [145]. Furthermore, to confirm and quantify the effect of vasoactive amines upon efflux, another distinct efflux pump substrate was used (Rh-6G) [103]. *Candida* cells treated with adrenaline showed a dose-dependent decrease of FUN-1 fluorescence intensity in comparison with non-treated cells (figure 1), thus indicating an increase in the efflux activity with a pEC_{50}= 5.81 ± 0.56. Although less pronounced, a similar effect was obtained with noradrenaline (pEC_{50}= 4.58 ± 0.22) (figure 1). Since isoprenaline at concentration up to 545 μM had no significant effect upon the efflux pumps activity it was possible to establish a rank order of potency: adrenaline>noradrenaline>>isoprenaline (figure 1). This rank order of potencies is achieved whenever an α-adrenoceptor-mediated effect is involved [288, 289]. Accordingly, cells treated with adrenaline showed a lower intensity of Rh-6G fluorescence than non-treated cells (figure 2). These results indicate the inhibitory effect of adrenaline on the accumulation of Rh-6G was concentration-dependent (figure 2). The same effect was observed with all the tested clinical isolates and with SC5314 strain.

A concentration-dependent decrease of FUN-1 accumulation in all strains was observed with medetomidine (a selective α_2-adrenoceptor-agonist) with a pEC_{50}= 7.83 ± 0.28 while phenylephrine (a selective α_1-adrenoceptor-agonist) in concentration up to 3 mM had no effect upon FUN-1 staining (figure 3). This result suggests that the effect of adrenaline upon *C. albicans* is mediated by a α_2-adrenoceptor, as only with medetomidine cells were able to reduce stain accumulation.

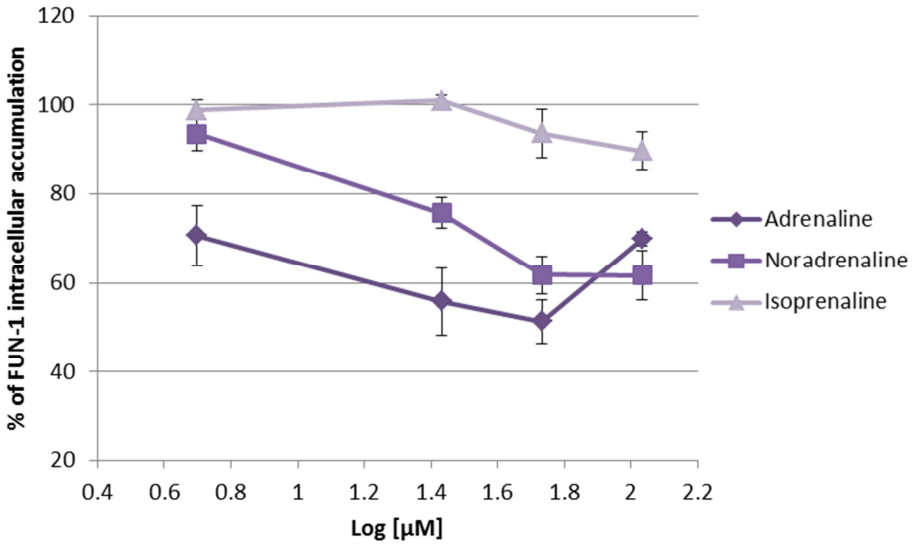


Figure 1. Effect of adrenaline (mixed α_1, α_2, β_1 and β_2), noradrenaline (mixed α_1, α_2 and β_1) and isoprenaline (selective β_1 and β_2) upon FUN-1 staining: a rank order of potencies was found (adrenaline>noradrenaline>>isoprenaline) showing a probable alpha mediated effect of adrenaline and noradrenaline on *C. albicans* clinical isolate (representative example). Data from three independent experiments is presented as mean ± standard deviation and shown as the percentage of FUN-1 accumulation relatively to untreated cells.

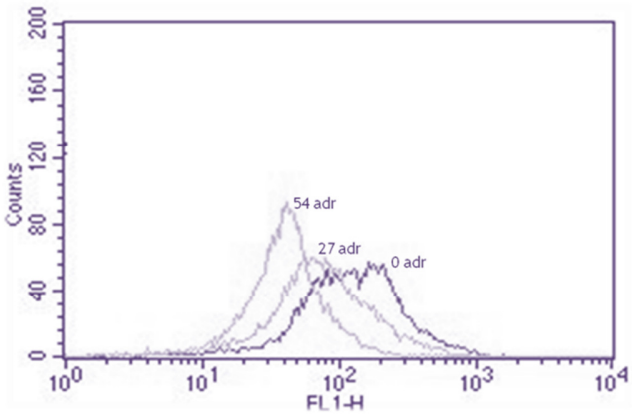


Figure 2. Effect of adrenaline (adr) upon Rh-6G staining: flow cytometric histogram representing the emitted fluorescence (Fl1) by a clinical isolate after 90 minutes of incubation with 0, 27 and 54 µM adr and stained with Rh-6G. A dose dependent reduction of Rh-6G accumulation is observed, shown by a decrease in the intensity of Fl1 fluorescence.

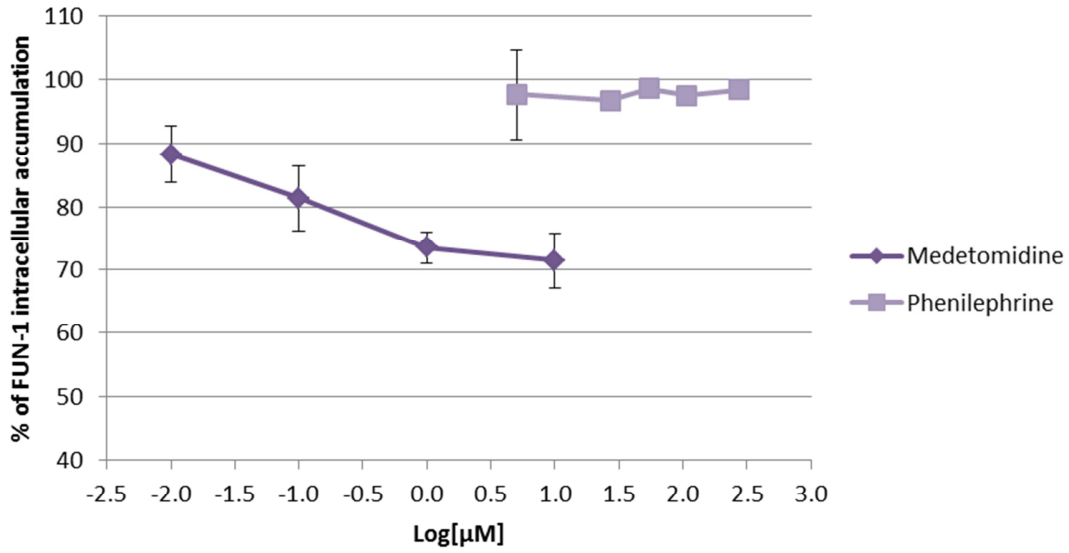


Figure 3. Effect of medetomidine (selective α_2 agonist) and phenylephrine (selective α_1 agonist) upon FUN-1clinical isolate staining. A dose dependent decrease of FUN-1 staining was caused by medetomidine, while phenylephrine showed a minor effect. Data from three independent experiments is presented as mean ± standard deviation and shown as the percentage of FUN-1 accumulation relatively to untreated cells.

Effect of adrenaline upon Rh-6G staining in CDR1 and/or CDR2 mutant strains

The results described strongly suggest that adrenaline acts as a promoter of efflux pumps activity. However to demonstrate the role of this effect in antifungal resistance, we assessed the effect of adrenaline upon the efflux by strains with diminished capacity to extrude since the genes encoding for the two most important efflux pumps had been deleted. Thus, strains without CDR1 (DSY448), CDR2 (DSY653) genes, and also both CDR1 and CDR2 (DSY654) were used.

As expected, in the DSY654 (*cdr2*Δ/Δ *cdr1*Δ/Δ) strain the effect of adrenaline was null and Rh-6G accumulated inside the cell. Conversely, in the parental strain a major reduction of Rh-6G staining was found. Intermediate result were observed in the DSY653 (*cdr2*Δ/Δ) and DSY448 (*cdr1*Δ/Δ) (figure 4). These results indicate that adrenaline acts at the CDR1 and CDR2 efflux pumps, stimulating Rh-6G extrusion.

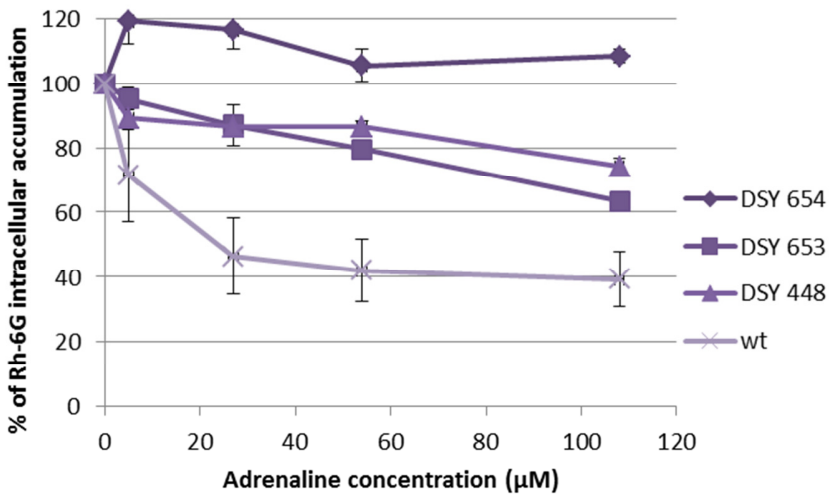


Figure 4. Effect of adrenaline on wild-type and *CDR1* and *CDR2* mutants strains. The percentage of accumulation of Rh-6G was higher in DSY 654 (*cdr1*Δ/Δ *cdr2*Δ/Δ) than DSY 653 (*cdr2*Δ/Δ) and DSY 448 (*cdr1*Δ/Δ). The wild-type strain *C. albicans* SC5314 showed a dose dependent decrease in Rh-6G accumulation. No decrease of staining was observed in DSY 654. All strains were treated with 0, 5, 27, 54, 108 µM of adrenaline for 90 minutes. Data from three independent experiences is presented as mean ± standard deviation and shown as the percentage of Rh-6G accumulation relatively to untreated cells.

Effect of adrenaline upon Rh-6G staining in cell receptor deleted strains

In order to identify and functionally characterize the binding target of adrenaline in *C. albicans*, selectively deleted membrane receptors strains were used and assayed for Rh-6G staining (figure 5). All strains showed a concentration- dependent decrease in the percentage of stain accumulation (figure 5A and C), except for the *ste3*Δ/Δ and *gpa2* Δ / Δ (NM8) strains in which treatment with adrenaline showed no effect (figure 5B and D, respectively). The increase of staining reveals the lack of Rh-6G efflux, contrary to the wild-type strain (P57072 and SC5314, respectively), especially at the higher adrenaline concentration tested (108µM). These results strongly suggest that adrenaline might act in *C. albicans* through the Ste3 mediated receptor and a Gpa alpha protein.

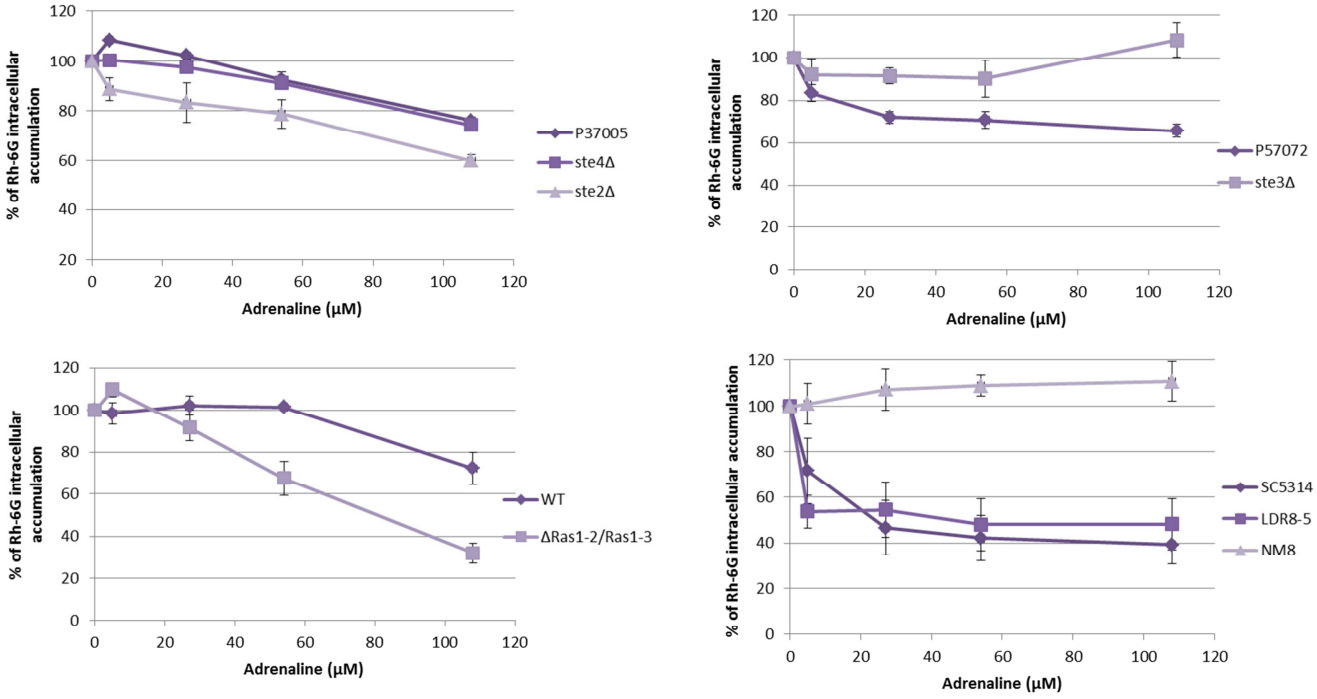


Figure 5 . Effect of adrenaline upon cell receptor deleted strains. A) strains P37005 (wild-type), *ste4Δ/Δ* and *ste2Δ/Δ* and C) strains *ΔRas1-2/Ras1-3* and respective wild-type (WT) all showed a dose dependent reduction of the % of Rh-6G accumulation. B) and D) Regarding *ste3Δ/Δ* and NM8 (gpa2 Δ/Δ) strains an increase of stain accumulation was observed distinct from the adrenaline effect observed on strain P57072 (WT) and LDR8-5 (gpr1Δ/Δ) and SC5314 (WT), respectively.

Effect of adrenaline upon CDR1 and CDR2 gene expression

In order to test whether increased extrusion promoted by adrenaline was due to the upregulation of *CDR1* and *CDR2* gene expression, immunoblotting assays were performed. Even after exposure to 54 µM of adrenaline for 90 minutes, no increased *CDR* expression was observed (Figure 6).

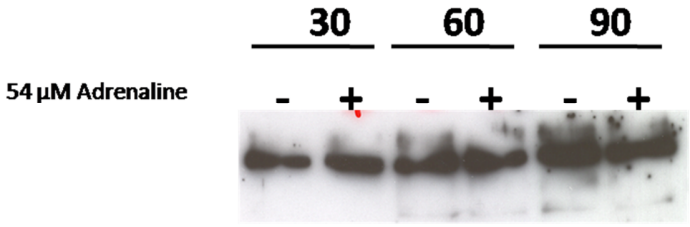


Figure 6. Immunodetection of Cdr1p in *C. albicans* strain SC5314. Cell extracts previously exposed (+) and not exposed (-) to adrenaline (54 µM) during 30, 60 and 90 minutes. No differences were found regarding Cdr1p detection.

Effect of adrenaline upon growth and oxygen consumption of SC5314 strain

CDR efflux pump activity is ATP-dependent. Thus, if adrenaline enhances its activity, high amounts of energy are required. The mitochondrial electron transport chain represents the most important cell apparatus for production of cellular ATP. Therefore the effect of adrenaline upon growth and oxygen consumption was investigated. We compared the growth rate of SC5314 strain in the presence of fluconazole with and without adrenaline (figure 7). Interestingly, a significant increase in the growth rate was observed after 14 hours incubation in the presence of adrenaline (figure 7).

Measurement of oxygen consumption in the presence of fluconazole and fluconazole plus adrenaline were carried out with *C. albicans,* strain SC5314. Oxygen uptake rate was higher in cells treated with adrenaline (p=0.016) (figure 8), indicating that adrenaline activates energy production. In the yeast cell, this energy promptly available could be directed to a response to the antifungal pressure and improve the fitness and stress response of the fungal population.

Taken together, these results demonstrate that adrenaline improves the fitness of a population under antifungal stress, activating energy production.

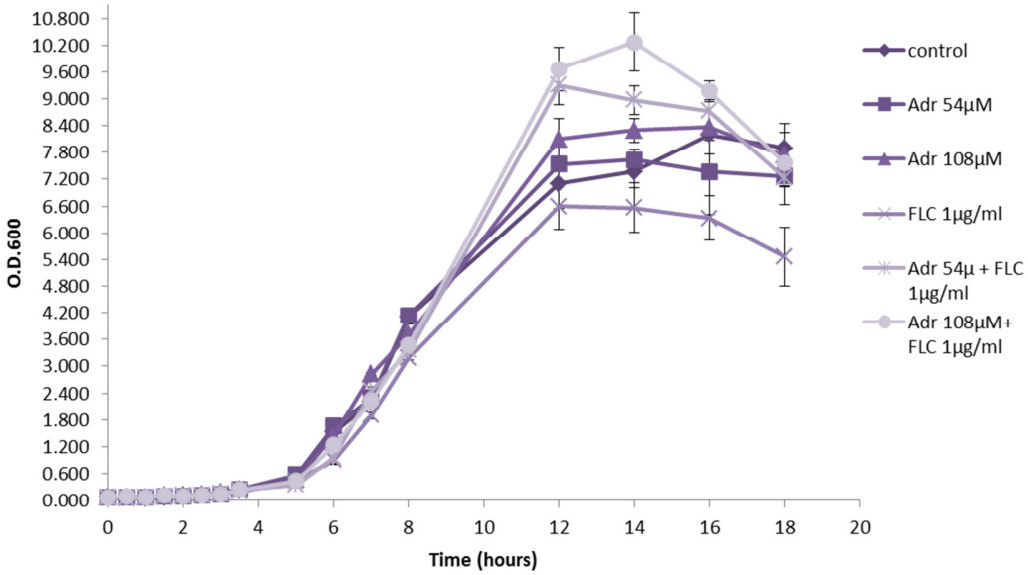


Figure 7. Effect of adrenaline (Adr) upon growth rate of *C. albicans* strain SC5314. Higher growth rates were observed whenever *C. albicans* was treated with Adr 108µM plus FLC 1µg/ml (Sub-MIC values). Significant differences (1µg/ml fluconazole *versus* 1µg/ml fluconazole plus 54µM adrenaline: p=0.030 and 1µg/ml fluconazole *versus* 1µg/ml fluconazole plus 108µM adrenaline: p=0.015) were observed following 14 hours incubation. Data from three independent experiences is presented as means of optical density (O.D.) ± standard deviation.

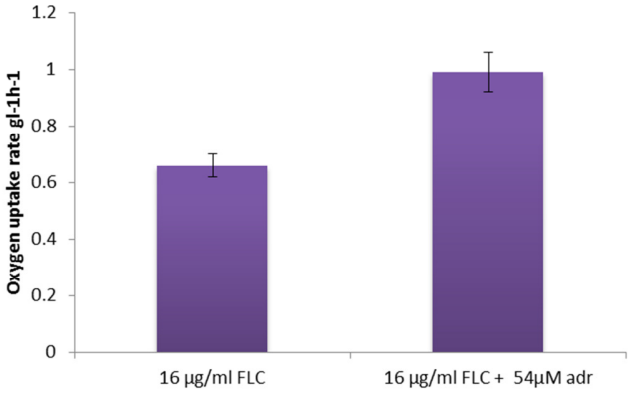


Figure 8. Effect of adrenaline upon oxygen consumption by *C. albicans* SC5314 strain. After 90 minutes in the presence of 16µg/ml fluconazole (FLC) or 16µg/ml fluconazole plus 54µM adrenaline (adr) the oxygen uptake rate was determined. Oxygen uptake rate was higher in cells treated with adrenaline (p=0.016). Oxygen consumption was measured by a Clark electrode. Data from three independent experiences is presented as mean ± standard deviation.

Discussion

It is well known that although *in vitro* microbial resistance usually correlates with clinical resistance, susceptibility *in vitro* is not invariably related to clinical success [290]. *In vivo* conditions do significantly differ from the *in vitro* ones, due to several factors, namely biofilm formation, yeast interaction with other microorganisms, and also due to *in vivo* exposure to non-antifungal drugs. Many drugs administered to critical care patients, like albumin and propofol, have the potential to interfere with the antifungal activity, by reducing the access, permeabilization or both of fungal cells [290-292]. In this study we showed that catecholamines, such as adrenaline and noradrenaline, two drugs widely used for life support, share the potential to interfere with antifungals namely by promoting tolerance or resistance to azoles.

We have addressed the interaction between catecholamines and antifungals using the checkerboard methodology and FUN-1 staining. Adrenaline and noradrenaline antagonized the effect of fluconazole, resulting in an increased fluconazole MIC values. The antagonistic effect observed between catecholamines and azoles, as well as the indifferent effect regarding amphotericin B and caspofungin, suggested a possible interference of adrenaline and noradrenaline on yeast cell efflux, the most common mechanism conferring resistance to azoles. In order to provide a better insight of this effect, FUN-1 flow cytometric analyses were performed. Besides information regarding metabolic activity FUN-1 also provides additional information about the mechanisms of azole resistance, being a good marker of efflux activity [145, 147]. The dose-dependent decrease of the fluorescence intensity of *C. albicans* cells incubated with adrenaline in comparison to non-treated cells suggested an increase of the efflux activity. With regard to the efflux of FUN-1 in presence of the other adrenoceptor agonists (noradrenaline, isoprenaline), a rank order of potencies became evident: adrenaline>noradrenaline>>isoprenaline. This ranking of potencies was very important finding since it allowed to understand the specificity of the mechanism like in mammalian cells [288]. In Human cells, whenever an α-adrenoceptor is involved, adrenaline and noradrenaline have higher affinity than isoprenaline [288]. This approach helped to typify the receptor involved in *C. albicans*. It suggests that adrenaline acts on the *C. albicans*

strains through an α-adrenoceptor. Selective α-adrenoceptor agonists were also tested (medetomidine α-2 agonist and phenylephrine α-1 agonist). The effect of medetomidine upon FUN-1 staining was similar to that obtained with adrenaline and noradrenaline, suggesting that the effect is mediated by an α-2 adrenoceptor [287]. Nevertheless more agonists and antagonists should be tested in order to better characterize the type of adrenoceptor involved in *C. albicans* cells. The results obtained with FUN-1 were confirmed with a more specific substrate, Rh-6G [103, 148]. The measurement of intracellular accumulation of Rh-6G is a useful method to identify azole resistance due to efflux pumps [103, 148]. In all clinical isolates a dose-dependent reduction of the Rh-6G staining was observed in the presence of adrenaline.

Yeasts cells have efflux mechanisms, like efflux pumps of the ABC transporter family (*CDR1* and *CDR2*) that reduce drug accumulation inside the cell. After assessing *cdr1*Δ/Δ and *cdr2*Δ/Δ strains, we could conclude that adrenaline promoted drug efflux, since the percentage of accumulation of Rh-6G was significantly lower than that found in the parental strain. Finally, adrenaline did not impair the staining of the double *cdr1*Δ/Δ *cdr2*Δ/Δ mutant. Taking together, these results demonstrated that adrenaline promotes the activity of *C. albicans* efflux pumps.

After uncovering the role of adrenaline as a contributor to antifungal resistance, we aimed to identify *C. albicans* surface receptors mediating such effects based upon analogies with mammalian cells. Several studies have reported the existence of GPCRs in yeasts, showing a GTP-binding site similar to those in eukaryotic G-proteins [277-281]. They comprise a highly diverse family of receptors which are involved in multiple cellular processes. In *C. albicans*, some GPCRs have been identified, being mainly involved in morphogenesis (Gpr1, Gpa2 and Ras) [277, 278] and mating (pheromone receptor- Ste2, Ste3 and Ste4) [279]. They mediate environmental stimulus responses that result in changes in cell physiology, morphology and adherence ability [293]. Considering the many homologies between yeasts and human cells, we raised the hypothesis that catecholamines could act on *Candida* through receptors similar to adrenoceptors that are present in eukaryotic cells. Adrenoceptors are GPCRs that evoke cellular signaling mechanisms after binding to catecholamines [294].

In an attempt to unveil the possible adrenaline receptor the effect of adrenaline upon Rh-6G staining was studied in GPCR-deleted *C. albicans* strains and compared with the corresponding wild-type strains. We could conclude that adrenaline acts through a pheromone receptor Ste3 and a Gpa alpha protein, since in strains *ste3*Δ/Δ and *gpa2* Δ / Δ (NM8) adrenaline did not impair the Rh-6G staining.

Aiming at uncovering the mechanisms underlying the increased efflux, immunoblotting assays were performed. The results revealed that adrenaline does not act as an inducer of the expression of CDR1 and CDR2 genes. However, adrenaline stimulates ATP production, and increases the activity of energy-dependent efflux pumps. In addition to increased efflux activity, other mechanisms can allow the cell to overcome antifungal drug induced stress [237, 295]. The mitochondrial electron transport chain has been implicated in drug tolerance or resistance and it is a very important cell apparatus for production of ATP [296-299].

In brief we found that adrenaline enhances drug efflux activity mediated by *CDR1* and *CDR2* pumps. This increase in efflux pump activity requires energy whose production is also stimulated by adrenaline. Interestingly, despite the presence of fluconazole, adrenaline can improve the fitness of fungal cells by stimulating energy production and fungal growth, thus allowing them to overcome the stress caused by the antifungal drug.

Overall, it is plausible to postulate that adrenaline can trigger ATP production through a GPCR by acting on the electron transport chain (via putatively a secondary messenger). In turn, these intracellular downstream events can increase the activity of efflux ATP-dependent pumps (*CDR1* and *CDR2*) and yeast cell growth, promoting concomitantly the tolerance or resistance to antifungal drugs.

Hospitals face at present an increasing emergence of resistant fungal pathogens. The knowledge on underlying mechanisms of induction of drug resistance by non-antifungal drugs like catecholamines will allow the development of new therapeutic strategies.

Part VII

In vivo synergistic effect between ibuprofen and fluconazole in *Candida albicans*

Background

Candida infections range from superficial mucocutaneous infections to life-threatening invasive infections. Especially the last one represents an increasing challenge for clinicians, with an attributable mortality around 40% [6, 7, 300]. The emergence and spread of antifungal resistance is one of the main factors that are responsible for this trend. Among azoles, fluconazole represented a landmark in the treatment of *Candida* bloodstream infections. However, its extensive use both for prophylaxis and therapy resulted in the emergence of fluconazole resistance [301-303].

The major mechanism responsible for high level azole resistance in *Candida albicans* clinical isolates is the overexpression of plasma membrane efflux pumps, the ATP-Binding Cassette (ABC) transporters, namely CDR pumps, or the major facilitator superfamily (MFS) transporters, MDR pumps [24, 27, 98, 101].

The knowledge of the underlying resistance mechanisms is of crucial importance since it could support approaches to achieve its reversion, thus leading to the development of new therapeutic strategies. Our previous studies showed that antifungal resistance conferred by increased efflux activity in *Candida* clinical strains could be reverted by ibuprofen [101, 145]. Ibuprofen ([2-(4-isobutylphenyl)-propionic acid]) is a non-steroidal anti-inflammatory drug (NSAIDs) used for its antipyretic, analgesic, and anti-inflammatory effects. In humans, it inhibits the synthesis and release of prostaglandins as mediators of inflammation. Ibuprofen acts synergistically with pyrazinamide, an tuberculostatic drug [182], azoles [101, 145, 183] and amphotericin B [184]. Previously, we had showed that ibuprofen acts synergistically with fluconazole, voriconazole and itraconazole, by inhibiting efflux pumps

mechanisms, thus promoting intracellular accumulation of [H^3]- itraconazole in *Candida* cells overexpressing *CDR1* and *CDR2* genes [101, 145].

Generally, azole exposure triggers several cellular and molecular mechanisms in *Candida albicans*, including the overexpression of genes related with antifungal resistance, mainly efflux pumps [118, 302, 304, 305]. With the development of new molecular biology methods, mainly DNA microarray technology, it was possible to get insights about the molecular basis involved in the evolution of antifungal resistance [23, 302, 306]. Herein we aimed to study the *in vivo* synergistic effect between these two drugs using a *C. albicans* systemic model of infection and to unveil the transcriptional profile involved in the reversion of fluconazole resistance by ibuprofen.

With this study we clarified the ability of ibuprofen to revert azole resistance, as well as elucidated about molecular changes associated with resistance reversion.

Material and methods

C. albicans strains and culture conditions

A *Candida albicans* blood stream isolate (CaS) susceptible to fluconazole, voriconazole and posaconazole was used throughout this study. In order to induce a resistant phenotype, a yeast suspension containing 10^6 cells in 10 ml of RPMI 1640 medium (RPMI 1640; Sigma, St. Louis, MO USA) was repeatedly incubated with fluconazole (FLC; Pfizer, Groton, CT, USA) at a final concentration of 16 μg/ml (therapeutic serum levels achieved during antifungal therapy) in order to induce a resistant phenotype (CaR) [307, 308]. Yeast suspensions were grown overnight at 35°C under agitation (150 rpm). Cultures were daily transferred to fresh medium with and without the same antifungal concentration for a total of 60 days. Each day 1ml aliquot from each subculture was mixed with 0.5ml of 50% glycerol and frozen at -70°C for later use. The Minimal inhibitory concentration (MIC) value of azoles was determined for the parent strain and the successive fluconazole exposed isolates.

Microdilution antifungal susceptibility testing

Assessment of the antifungal susceptibility profile was performed according to the CLSI M27 A3 protocol in RPMI 1640 culture medium (Sigma)[33]. The minimal inhibitory concentration (MIC) of FLC, voriconazole (VRC; Pfizer) and posaconazole (PSC; Schering-Plough, NJ) was determined [33]. Strains were classified as susceptible (S), susceptible-dose dependent (S-DD) or resistant (R) to azoles according to the breakpoints defined by CLSI [33]. Although definitive breakpoints have not yet been established for posaconazole, a MIC ≤1 μg/ml was considered susceptible.

Effect of ibuprofen upon azole resistance

In order to study the effect of ibuprofen (Ibu; Sigma) on the resistant phenotype displayed by CaR strain, MIC value of FLC, VOR and PSC was re-determined as described above in the presence of 100 μg/ml of ibuprofen, a concentration previously described to impair azole extrusion [101, 145].

In vivo effect of ibuprofen upon fluconazole resistance

All *in vivo* investigations were performed in the animal facility of the Cardiovascular Research & Development Unit Faculty of Medicine, University of Porto, in accordance with the European Directive 86/609, transposed to the Portuguese Law by DL 129/92 and by Portaria 1005/92. A murine model of disseminated candidosis was used to study the effect of ibuprofen upon fluconazole resistant phenotype. Female specific-pathogen-free BALB/c mice (age, 6 to 8 weeks; weight, 17 to 20 g; Charles River Laboratories, Barcelona, Spain) were housed in microisolator cages with five animals per group, having access to food and water *ad libitum*. To induce disseminated infection mice were injected with $5x10^5$ cells of the CaS or the CaR strain in 0.1ml of sterile saline via the lateral tail vein. The fluconazole effective dose in reducing the pathological effects of i.v. *C. albicans* challenge relative to untreated mice (control) was defined as the 50% effective dose (ED_{50}) [309]. Therapy was initiated 3 hours after yeast challenge and was administered intraperitoneally once a day for a total of 4 days. Mice were treated with FLC (8 to 60 mg/kg of body weight/day), with Ibu (10 or 20 mg/kg/day) [182, 310] or FLC (8 to 60 mg/kg of body weight/day) + Ibu (10 or

20 mg/kg/day). Mice weight was daily registered and at day 4 post-infection mice were euthanized and the kidneys were aseptically removed. The right kidney from all mice was homogenized in 5 ml of phosphate buffer saline (PBS; Sigma) and weighted. Serial dilutions of the homogenate were plated onto YPD agar in triplicate and incubated at 30°C for 48 hours. The fungal burden was calculated as the number of colony forming units (CFU) per gram of tissue. The kidney fungal isolates were frozen in YPD broth with 50% glicerol at -70°C for later MIC azole determination according CLSI M27 A3 protocol [33]. For histological studies the left kidneys were fixed in 10% phosphate-buffered formalin, embedded in paraffin, sectioned, and stained with periodic acid-Schiff (PAS) stain. Slides were observed under a Zeizz Axioskop 40 microscope and acquired with an Axiocam MRc5 Zeizz.

RNA extraction

Strains CaS and CaR were grown in 50-ml YPD broth at 180 rpm and 30°C until an optical density at 600nm of approximately 0.8 was reached. CaR strain was grown in the presence of 16 µg/ml of FLC (CaRFLC) and of 16 µg/ml of FLC plus 100 µg/ml of ibuprofen (CaRFLCIbu). Total RNA was extracted using the hot acid phenol method, as described by Köhrer & Domdey [311]. RNA concentration was assessed by Nanodrop ND-1000 and the RNA quality and integrity levels were controlled by capillary electrophoresis in the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA), according to the manufacturer's instruction. Only samples yielding a 28S rRNA/18S rRNA ratio ranging from 1.6 to 2.2, with a "RNA Integrity Number" (RIN) higher than 7.0 and showing the absence of degradation were used. RNA samples were stored at -70°C for later use.

Probe preparation and microarray hybridization

All the experiments were carried out using *C. albicans* microarrays manufactured by Agilent Technologies. A RNA/primer mix containing 50 µg RNA, 1.25 µg Oligo dT_{12-18}, (Invitrogen, Carlsbad, CA) was incubated at 70°C for 10 minutes (min) and chilled on ice for 5 min. Then, a labeling mixture containing dGAC – mix 0.1mM, dTTP 0.03mM, aa-dUTP 0.08 mM, first strand buffer 1x, DTT 0.01M, Superscript II Reverse Transcriptase 400U (Invitrogen) was added and the reaction was incubated at 42°C for 60min. Residual RNA template was

degraded by hydrolysis. Briefly, the reaction mixture was incubated at 95°C for 2 min and placed on ice immediately; 10 µl NaOH 1M (Sigma) and 10 µl EDTA 0.5M (Sigma) were added to the reaction and incubated at 65°C for 15min. Afterwards, 25 µl HEPES buffer 1M (pH 7.5, Sigma)were added. Samples were purified using the Microcon - 30 (Amicon Microcon YM-30, Millipore) columns, according to the manufacturer's instructions and were coupled to Cy3 and Cy5 fluorophores. Before hybridization, free dyes were removed using Chromaspin-30 (Clontech) columns, the efficiency of cDNA synthesis and dye incorporation was measured spectrophotometrically in the Nanodrop ND-1000. For full slides, 200 ng of Cy3/5 labeled sample were used. Hybridization was performed according to the labeling kit Quick Amp Labeling for Two-Color Microarray-Based Gene Expression Analysis protocol (from Agilent Technologies). A common reference design with dye-swap replicates was used. Total RNA obtained from CaS strain was used as hybridized against samples studied. Control of experimental background was performed through self-self hybridization; a total of four experiments were made.

Image acquisition and data processing

The microarrays were scanned with an Agilent G2565BA microarray scanner and the fluorescence intensities were obtained with Agilent Feature Extraction Software Protocol GE2. Pre-processing of the data was performed using the Biometric Research Branch (BRB)-ArrayTools v3.4.0 software.

Statistical analysis and functional annotation of the data

For data analysis the statistical software R and the package Limma from Bioconductor (www.bioconductor.org) were used. Microarray normalization was performed using the method *loess* and within arrays quantile [312]. After normalization, median gene expression was determined for each ORF. Relative gene expression and statistically significant differences found between the different samples were determined with the software Limma package. Significant differences were encountered using the Empirical Bayes

statistical test [313]. Hierarchical clustering of the normalized and dye swap averaged samples was performed using the method *complete linkage*.

Only probes with a fold change (FC) greater than 2 and adjusted $p<0.05$ were used in analyses. Gene ontology identification was performed for significant differentially expressed genes using the Web tool GoTermFinder available on the website of the Candida Genome Database (http://www.candidagenome.org/cgi bin/GO/goTermFinder).

Quantitative RT-PCR

In order to confirm microarray data, cDNA was synthesized from 100ng in 20µl of total RNA from CaS, CaRFLC and CaRFLCIbu strains using a Superscript III reverse transcriptase kit (Invitrogen) according to manufacturer`s instructions. Quantitative real- time PCR was performed using PerfeCTa SYBR green Fast Mix (Quanta Biosciences) on a Realplex Mastercycler instrument (Eppendorf, Madrid, Spain) and according to the following program: 1 minute hot start at 95°C, a 35-cycle program composed of 15-seconds (s) denaturation step at 95°C, a 30-s annealing step at 60°C, and a 30-s extension step at 60°C, ending by a 5-min final extension step at 60°C. The primers used were designed using OligoExplorer program (listed in Table 1). *ACT1* was used as the normalization gene.

Table 1. Sequences of primers used in RT-PCR

Primer name	Primer sequence
CDR11 - F	**5´-GGTCACGAATCTACTTTGGAA-3´**
CDR11 - R	5´-CGGGTCTCATAATGGCAT-3´
NAG3 - F	**5´-CAAGGGGGTGGAAAAGAT-3´**
NAG3 - R	5´-TCGTTGTAGTCAGTGTGTGG-3´
MDR1 - F	**5´-CCCGAAAACCCTCAAAAT-3´**
MDR1- R	5´-CGACTCTTCCAATACCAAAATC-3´
ERG1 - F	**5´-GACGAGACCATTACTATCCCTT-3´**
ERG1 - R	5´-TTACACCATCAACGGCAT-3´
ACT1 - F	**5´-ATGGACGGTGAAGAAGTTG-3´**
ACT1 - R	5´-CAAGAGATGGGAAAACAGC-3´

Results

In vitro induction of resistance

The repeated exposure of the susceptible strain (CaS) to therapeutic serum concentrations of FLC the acquisition of an azole cross-resistant phenotype to FLC, VRC and PSC (table 2). Table 2 resumes MIC values assessed following 60 days of incubation with FLC.

Effect of ibuprofen in azole susceptibility

The azole susceptibility pattern of the parental (CaS) and the azole resistant induced strain in the absence and presence of ibuprofen is detailed in table 2. The MIC values decreased in the presence of Ibu for all strains, the phenotype changing from R to S regarding all the tested azoles (table 2); meanwhile, the MIC values remained unchanged regarding the susceptible strain (table 2).

Table 2. Minimal inhibitory concentrations (MIC) and phenotypes of the *Candida albicans* parental susceptible (CaS) and resistant (CaR) strain after exposure to fluconazole (FLC) to FLC, voriconazole (VRC) and posaconazole (PSC) alone and in combination with subinhibitory concentrations of ibuprofen (Ibu; 100 μg/ml). MICs were determined according the CLSI M27-A3 protocol. Susceptible, susceptible dose dependent and resistant phenotypes are represented as S, S-DD and R, respectively.

	MIC (μg/ml)/ phenotype					
Strains	**FLC**	**FLC+Ibu**	**VRC**	**VRC+Ibu**	**PSC**	**PSC+Ibu**
CaS	1/S	1/S	0.06/S	0.06/S	0.03/S	0.06/S
CaR	>64/R	4/S	>8/R	0.06/S	>8/R	0.125/S

In vivo synergistic effect between FLC and Ibu

In order to investigate the potential clinical application resulting from the synergistic effect between FLC and ibuprofen experiments were conducted in a murine candidosis model. The fungal burden of CaS and CaR strains in mice kidneys were determined after treatment for 4 days with 8 to 60 mg/kg of body weight/day of FLC alone or combined with 10 or 20 mg/kg of body weight/day of ibuprofen. On mice infected with the CaS strain and treated with 30 mg/kg of body weight/day (ED_{50}) of FLC a significant reduction (p<0.001) of CFU/g of kidney was found when compared with untreated mice (figure 1). On mice infected with the CaR strain no significant reduction on fungal burden was achieved even when treated with 60 mg/kg of FLC. The group of mice infected with CaR strain treated or untreated with FLC showed the highest weight loss during at the first and at the fourth day of infection (figure 2). However, when FLC was associated to the two concentrations of ibuprofen, a significantly reduction of CaR fungal burden (p<0.001) was observed, especially in mice treated with 30 mg/kg of FLC plus 20 mg/kg of ibuprofen (figure 1). These mice also showed weight increase at the first and at the fourth day of infection (figure 2).

Interestingly, the yeast isolates recovered from mice kidneys retained the susceptible and the resistant phenotype as displayed previously to infection with CaS and CaR strains, respectively. Azoles MIC values remained unchanged.

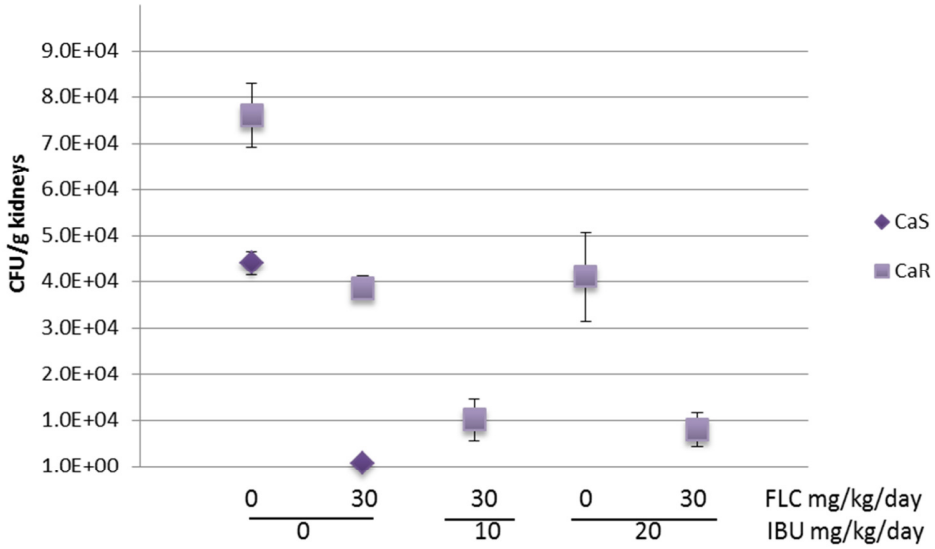


Figure 1. *In vivo* antifungal synergistic effect between fluconazole and ibuprofen against *C. albicans* systemic infection. Mice infected with the parent susceptible strain (CaS) and the induced resistant strain (CaR) were treated with 30mg/kg/day of fluconazole (ED_{50}) (FLC), and 10 or 20 mg/kg/day of ibuprofen (Ibu) intraperitoneally during 4 days. The right kidneys (from 5 mice per group) were removed, homogenized, weighed, serially diluted and plated onto YPD agar plates. Colony forming units (CFU) per gram of kidney were determined and plotted as mean value and respective standards error.

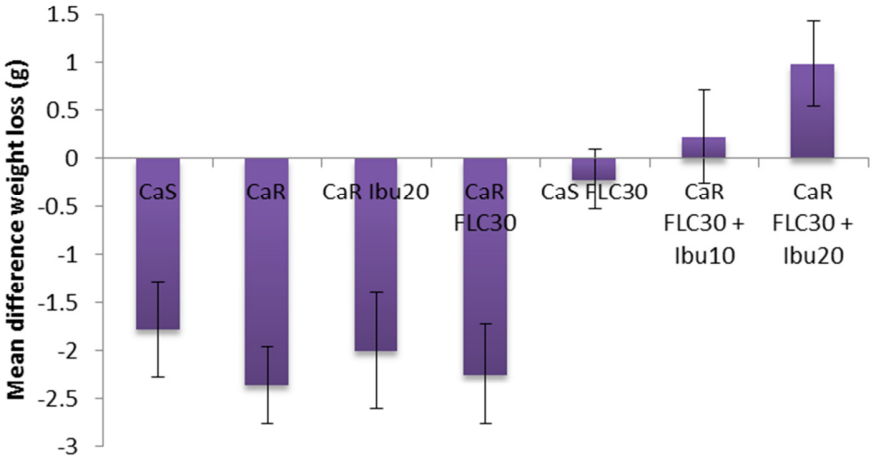


Figure 2. Effect of the combination of fluconazole plus ibuprofen on mice weight loss during *C. albicans* systemic infection. Mice infected with *C. albicans* susceptible (CaS) and the resistant (CaR) strains and treated and untreated with 30mg/kg/day of fluconazole (FLC30) and 10 or 20 mg/kg/day of ibuprofen (Ibu10 and Ibu20, respectively) intraperitoneally during 4 days were daily weighted. Differences in weight loss between the first and the fourth day of infection are plotted as mean values plus the respective standard errors.

Regarding the PAS stained histopathology sections of mice kidneys, susceptibility of mice to infection with the resistant strain (CaR) was evident on untreated or FLC treated mice. At day four post infection PAS staining of untreated mice infected with CaR strain revealed a dramatic increase in fungal cell numbers, both blastoconidea and hypha morphotypes (figure 3A) similarly to the findings in mice treated with 30 mg/kg of FLC (figure 3B, C, D and F). In mice infected with CaR strain and treated with 30 mg/kg of FLC plus 20 mg/kg of ibuprofen rare yeasts were found, all showing the blastoconidea form (figure 3 F, G). These results were in agreement with CFU counts obtained in fungal burden assays.

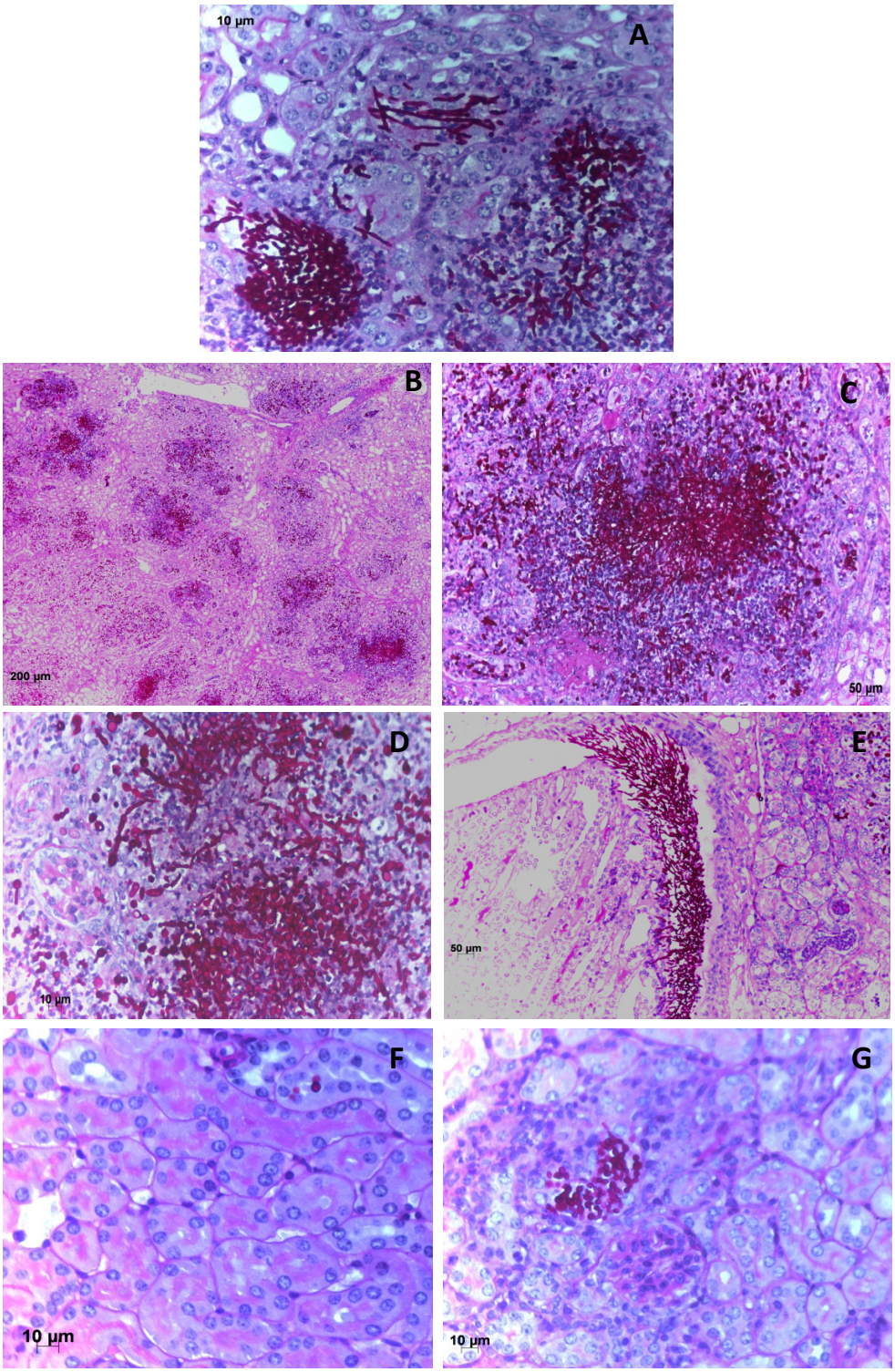


Figure 3. Representative example of kidney histology slides of PAS-stained paraffin sections of kidneys recovered from mice infected with $5x10^5$ cells/0.1 ml of *C. albicans* resistant (CaR) strain at day four post-infection, untreated (A; 40x amplification), treated with 30mg/kg/day of FLC (B, C, D, E; 10x, 20x, 40x and 20x, respectively) and treated with 30mg/kg/day of FLC plus 20 mg/kg/day of Ibu (F, G; 40x).

Microarray transcriptional profile

Microarray assays allowed the evaluation of the expression of 6158 *C. albicans* genes, however only the differentially expressed genes ($p < 0.05$) were used in analyses. The gene expression of the induced fluconazole resistant strain pre-exposed to FLC (CaRFLC) was compared with the parent susceptible CaS strain and 836 genes were differentially expressed, of which 207 were upregulated and while 629 were downregulated. The biological processes encoded by the majority of these genes are unknown (30% of upregulated genes and 18% of down regulated genes) (figure 4). However up to 5% of the upregulated genes are associated with transport processes and response to chemical stimulus. A large number of such genes are involved in antifungal resistance mechanisms, mainly CDR and sterol biosynthesis genes.

When compared with the parent strain, the presence of Ibu plus FLC (CaRFLCIbu) altered the expression of 1517 (411 upregulated and 1106 down regulated) (figure 5). Ibuprofen exposure upregulated genes related with transport, response to chemical stimulus and cell wall/membrane organization processes (figure 5). A minority of such genes was related to protein folding (1%) and transduction (1%) (figure 5). Genes involved in cell cycle (5%), RNA (9%) and DNA (5%) metabolic process were differentially downregulated (figure 5).

Figure 6 describes the transcriptome analysis of the induced FLC resistant strain (CaRFLC) and after exposure to fluconazole plus ibuprofen (CaRFLCIbu). The CaRFLC strain showed overexpression of *ERG251, CDR1*, *CDR4, CDR11 and UPC2* genes. However, in the presence of fluconazole plus ibuprofen a significantly decrease of *CDR11* and *ERG251* gene expression and of the transcription factor *UPC2* was found (figure 6). *MDR1*, *CDR4*, *NAG3* and *NAG4* genes showed increased expression in the presence of fluconazole plus ibuprofen, resulting in significant differences when compared to strain CaRFLC (figure 6). The microarray results were confirmed with quantitative real-time PCR (figure 7). Significant different levels of gene expressions was observed between CaRFLC and CaRFLCIbu strains ($p<0.05$).

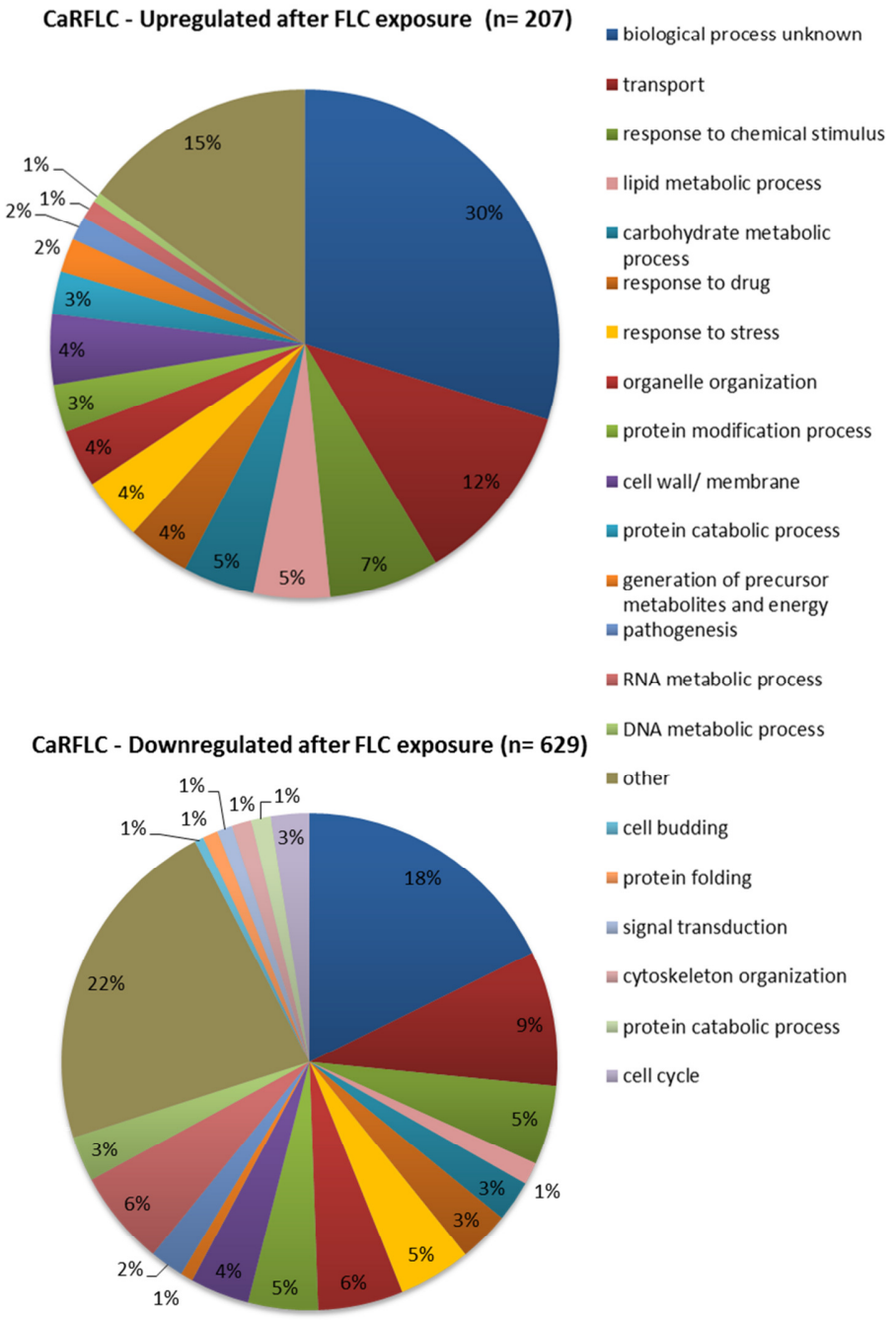


Figure 4. *C. albicans* genes up-regulated and downregulated after fluconazole (FLC) exposure (*C. albicans* resistant - CaRFLC) *in vitro* comparatively to gene expression in unexposed cells (*C. albicans* susceptible parent strain-CaS) grouped according to their biological processes.

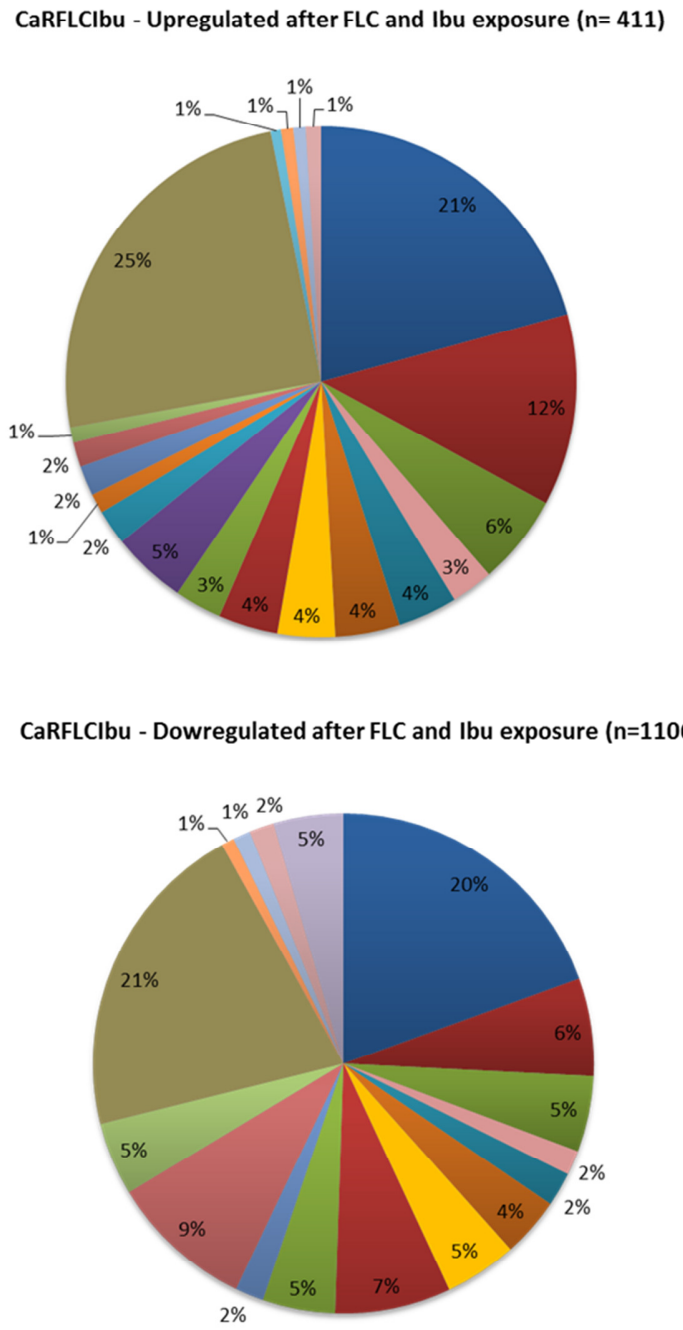


Figure 5. *C. albicans* genes up-regulated and down regulated after fluconazole (FLC) and ibuprofen exposure (CaRFLCIbu) comparatively to gene expression found in unexposed cells (*C. albicans* susceptible parent strain-CaS) grouped according to their biological processes.

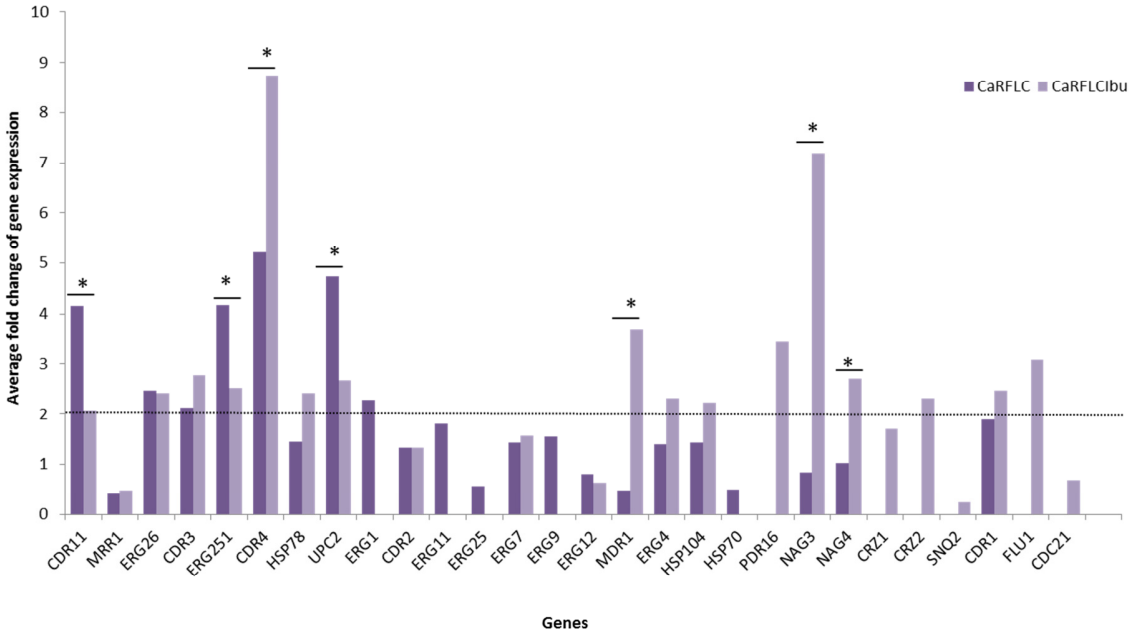


Figure 6. Microarray analysis of the genes classically involved in antifungal resistance of the resistant strain (CaRFLC) and of the resistant strain following exposure to ibuprofen (CaRFLCIbu). Bars indicate the average fold change of significantly expressed genes. Only genes with fold change> 2 were considered upregulated. (*p<0.05 when comparing between average gene expression of CaRFLC and CaRFLCIbu strains).

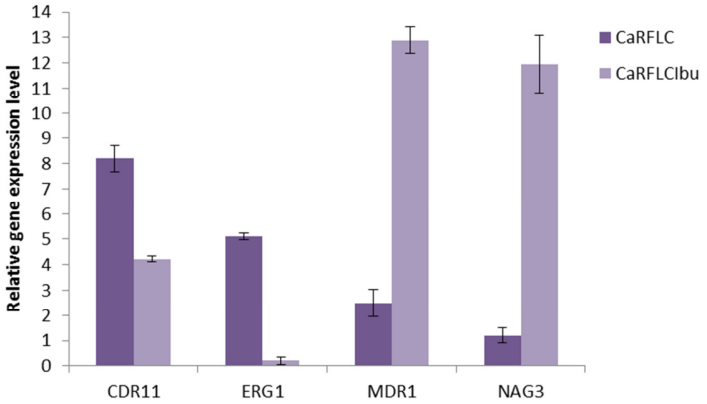


Figure 7. Quantitative real time PCR analysis of genes implicated in antifungal resistance whose expression was found to be altered in microarray assay. CaRFLC, *C. albicans* fluconazole resistant strain; CaRFLCIbu, *C. albicans* fluconazole resistant induced strain exposed to 16µg/ml fluconazole plus 100 µg/ml of ibuprofen. Bars represent the average gene expression relative to the susceptible CaS strain.Five independent experiments were performed. A significantly different gene expression (p<0.05) was found between CaRFLC and CaRFLCIbu strains, for all represented genes.

Discussion

A high mortality rate is often observed in immunocompromised patients with systemic fungal infections, despite being often prescribed long courses of antifungal therapy. In addition, antifungal therapeutic options are limited, being the azoles the most commonly used drugs [314]. The widespread use of fluconazole, especially for prophylaxis, has selected *Candida* species with easily inducible resistance, such as *C. glabrata* [315] and *C. tropicalis* [306]. The development of resistance by *C. albicans* after *in vitro* fluconazole exposure has been widely demonstrated [302, 304, 305]. Aiming to reproduce *in vivo* findings occurring during antifungal treatment, we daily exposed a susceptible *C. albicans* strain to serum concentration levels of fluconazole. As expected fluconazole exposure resulted in the development of cross resistant profiles to azoles.

The major mechanism described as responsible for azole resistance in clinical *Candida albicans* isolates is overexpression of plasma membrane efflux pumps [22, 96, 117]. The use of DNA microarray technology push forward the knowledge of the protagonists involved in cellular processes [23, 96, 152, 153, 302, 304, 306, 315]. The transcriptional responses of *C. albicans* after fluconazole exposure showed an increased expression of genes encoding CDR efflux pumps as well as of genes involved in the ergosterol biosynthesis, as expected [96, 117, 302, 304, 305]. Similarly to *C. albicans*, species like *C. glabrata*, *C. tropicalis* and *C. parapsilosis* also use efflux pumps as the main tool for the development of azole resistance [153, 306, 315].

During the recent years several approaches have been used in order to overcome the development of antifungal resistance. The inhibition of antifungal extrusion by blocking specifically ABC efflux pumps is a possible way to impair multiple drug resistance. The use of antifungals which are not substrates of efflux pumps, of pump blockers that inhibit the antifungal extrusion (inhibiting H^+ ATPase reducing the energy required for efflux activity) or the increase of antifungal uptake rate in order to maintain a high intracellular concentration of the drug are all strategies to achieve a high concentration of the antifungal compound at its site of action [22]. The pursuit of knowledge of efflux pumps mechanism in *Candida*

arises from the homology between yeasts and human cells. In eukaryotic neoplasic cells, ATP-dependent drug efflux pumps, such as P-glycoprotein (P-gp) which is encoded by MDR1 gene, are important mediators of resistance, contributing to failure of cancer therapy. Over the years different strategies were undertaken in order to overcome drug resistance by efflux, including the development of agents able to modulate P-gp activity, such as pump substrates like FK506 [176] or cyclosporine A [177], calcium channel blockers like verapamil [316] or anti-malaric analogs [317].

Azza *et al* demonstrated that ibuprofen could inhibit methotrexate efflux transporters in the human kidney [318]. A similar effect was described regarding FK506 (tacrolimus), a potent immunossuppressor agent used for the prevention of allograft rejection and as a calcineurin inhibitor, shows a synergistic effect when combined with antineoplastic agents on tumor cells, decreasing or even suppressing multidrug resistance by competing with cytotoxic drugs for the P-glycoprotein [319-321]. Thus, a similar approach could be applied to *Candida albicans* cells, using non- antifungal drugs exhibiting a synergistic effect with antifungals that could induce a reversion of resistance. In *Candida*, FK506 has shown to be a potent inhibitor of the calcineurin pathway, rendering the normally fungistatic azole effect to fungicidal [169, 179]. The calcineurin pathway has been shown to be critical for survival and stress responses in several fungi, including the *in vitro* antifungal activity against *Saccharomyces cerevisiae*, *C. albicans* and *Cryptococcus neoformans* [169, 322].

In the present study, a decreased of the MIC levels for the three azoles tested in the presence of ibuprofen was registered in strain CaR, changing the phenotype from azole resistant to a susceptible one. The synergism between posaconazole and ibuprofen was hereby demonstrated for the first time.

The *in vivo* assays clearly demonstrated that ibuprofen acts synergistically with fluconazole in mice infected with the CaR strain. A significantly reduction of CFU kidney counts were obtained in mice treated with both drugs, as well as a recovery of mice weight. These results were attested by the histological assays. A dramatic increase in fungal cell numbers was observed in the kidneys of mice infected with CaR strain untreated or treated with

fluconazole. However, fluconazole in combination with ibuprofen promoted fluconazole activity, histologically showing scarce fungal cells.

C. albicans cells recovered from mice treated with fluconazole plus ibuprofen still displayed a resistant phenotype to fluconazole. Consequently, we can conclude that the presence of ibuprofen is mandatory for the reversion of the azole resistance.

Trying to scrutinize the molecular base involved in the ability of ibuprofen to revert antifungal resistance we used microarray analysis of CaRFLC comparing the transcriptional profile to CaRFLCIbu strain. The CaRFLC strain revealed an overexpression of ERG and CDR genes, as expected [302, 304, 305]. The presence of ibuprofen significantly increased the expression of CDR and MDR efflux pumps (*CDR4*, *MDR1*, *NAG3* and *NAG4*). Interestingly, these findings may be seen as cellular salvage attempt mechanisms to overcome the inhibitory capacity of ibuprofen. Yeasts cells have the capacity to trigger cellular salvage pathways when facing stress conditions like in the presence of antifungals or other drugs [22, 160, 237]. Similarly, regarding echinocandins, the ability to grow at high caspofungin concentrations and has been suggested to relate to a compensatory increase in cell wall chitin [128, 130]. This salvage mechanism strengths cell wall damaged by exposure to echinocandins.

MRR1 and *TAC1* are all transcription factors of *MDR1* and *CDR* genes [96, 118, 152, 305, 323-325]. Despite the significative increase of *MDR1* gene expression displayed by CaRFLCIbu strain, *MRR1* expression remained downregulated and no difference was seen between exposed and non-exposed isolates to ibuprofen. On the other hand, the presence of ibuprofen promoted the downregulation of *CDR11*, *ERG251* and and the transcription factor *UPC2*. In fact, these results strongly suggest that ibuprofen could act at the transcriptional level. However, and due to the contradictory gene expression profiles found and their respective transcription factors, this assumption still needs further investigation.
Overall, our results stress the fact that ibuprofen can inhibit efflux pumps, either by blocking access to the binding site or by blocking the expression of the pumps. Besides, since it is not immunosuppressive, this anti-inflammatory drug has advantages over FK506.

Further studies are being addressed in order to uncover the main mechanism of ibuprofen inside the yeast cell as well as to assess its influence in the dynamics of the induction of antifungal resistance.

By allying anti-inflammatory and analgesic properties, ibuprofen in combination with fluconazole could play a relevant role in a therapeutic strategy for severe fungal infections.

CHAPTER IV

Conclusions and Future Perspectives

Conclusions

Over the last 30 years medical advances led to a significant increase of life-threatening fungal infections. The incidence and mortality rates associated with invasive candidosis have remained unchanged for more than a decade despite the advances in the field of antifungal therapy. Such infections could be treated more effectively if faster and more specific diagnostic and therapeutic approaches were available. Preventive safe strategies targeting patients with a high-risk profile, the development of new diagnostic tools for early identification of fungal species, including innate resistant species or those that are more prone to develop multidrug resistance, especially in patients submitted to long term therapy are of utmost importance. In conjunction, preventive attitudes should be imperatively implemented in order to reduce the number of health care related infections. The comprehension of the routes of transition is essential to overcome this objective.

Given the association between antifungal exposure and the development of resistance, prophylaxis must be selectively restricted to high-risk patients. Apparently there is no class of antifungal agents that is immune to the development of acquired resistance. It is essential that laboratories start performing routinely *in vitro* susceptibility testing especially in isolates from invasive infections, isolated from patients receiving antifungal prophylaxis and in strains isolated from patients who do not respond to therapy.

Intricate signaling networks govern the development, morphogenetic transitions but also the evolution of antifungal drug resistance in *Candida*. Cell stress may be caused by other concomitant factors apart the presence of the antifungal drug. Medication often administered to critical care patients may trigger a medley of escapes responses in order to ensure survival which may also be responsible for the discrepancy between *in vitro* and *in vivo* susceptibility profile. The knowledge of the mechanisms involved in antifungal resistance will help to design effective measures to reverse it. Ibuprofen may represent a hopeful compound in the reversion of azole resistance by efflux activity.

Regarding echinocandins, point mutations in *FKS* genes are responsible for the decreased susceptibility; such findings stress the need for the development of novel molecules with higher affinity to the target.

In brief, the research presented has led to the following findings:

Following the assessment of the genetic relatedness between simultaneous and/or successive *Candida albicans* isolates from fungaemia patients and of the antifungal susceptibility profile (detailed in Part I) the following conclusions were obtained:

- *C. albicans* colonization and/or infection of different body sites may represent a predisposing condition or an initial step towards the subsequent fungaemia development;
- The resistance of blood infection by successive isolates with similar genotypes suggests the failure of antifungal therapy;
- Isolates displaying the same or similar genotypes were obtained from patients who shared the same hospital department, suggesting the nosocomial origin of the infection in such cases;
- The induction of azole resistance was observed in similar strains from different patients admitted in distinct periods of time, suggesting that the strains were endemic to the hospital environment;
- Hospital acquired bloodstream infections are linked with a higher risk of antifungal resistance, thus needing close monitoring.

The novel flow cytometric protocol developed to measure yeast cell wall chitin (detailed in Part II) helped to understand the effect of echinocandins on fungal cell wall:

- Flow cytometry protocol showed to be a simple and reliable assay to accurately quantify cell wall chitin in yeast cells;

- No relationship between chitin content and the caspofungin susceptibility profile was found;
- The accuracy of this novel simple and reliable methodology can predict the occurrence of the paradoxical effect, representing a valuable tool for the detection of antifungal compensatory mechanisms in the presence of high echinocandin concentrations.

In Part III the first case report describing acquisition of echinocandin resistance by *C. glabrata* following anidulafungin treatment was addressed. The main conclusions were:

- The acquisition of resistance to anidulafungin was associated with an increase in caspofungin and micafungin MIC value, suggesting the development of cross-resistance between these three echinocandins drugs;
- No relation between chitin amount and antifungal resistance was established;
- Structural alterations in the HS1 of *FKS*2 molecule due to the Ser663Pro substitution and a Phe659 deletion lead to a dramatic decrease of anidulafungin efficacy *in vivo*.

Studies related to the contribution of mitochondrial respiration in the intrinsic fluconazole resistance displayed by *C. krusei* (Part IV) revealed that:

- An alternative respiratory pathway, cyanide-resistant, was described for the first time as a characteristic of *C. krusei* species;
- Such alternative pathway due to an alternative oxidase is unrelated to fluconazole resistance; nevertheless it protects *C. krusei* from oxidative stresses;
- The alternative respiratory pathway is a potential target that should be taken into account considering the development of new therapeutic strategies in the case of *C. krusei* infections.

The research described in Part V provided an opportunity to describe the effect of propofol in antifungal drug resistance. The main conclusions were:

- The promotion of antifungal resistance in presence of propofol lipidic emulsion results from a reduced access and/or permeabilization of the antifungal agents into the fungal cells;
- The major responsible for such effect was the lipidic vehicle of propofol emulsion.

The assessment of the effect of adrenaline upon *C. albicans* (Part VI) has led to the following conclusions:

- Adrenaline enhances drug efflux activity mediated by *CDR1* and *CDR2* pumps;
- Despite the presence of fluconazole, adrenaline promotes the fitness of fungal cells by stimulating energy production and fungal growth, thus allowing the overcome of stress caused by the antifungal drug;
- Adrenaline acts on *C. albicans* cells through a pheromone receptor Ste3 and a Gpa alpha protein.

Part VII addressed the *in vivo* synergistic effect between ibuprofen and fluconazole. This study provided the following conclusions:

- The *in vivo* assays clearly demonstrated that ibuprofen acts synergistically with fluconazole in mice infected with a *C. albicans* resistant strain;
- The presence of ibuprofen is mandatory for the reversion of fluconazole resistance.

This research resulted in a more comprehensive understanding of the multiple mechanisms that can influence antifungal resistance or tolerance. The deliverables of our studies include a better awareness of the efforts that should be made for the implementation of routine

antifungal susceptibility testing and provide new hope for the prevention of azole antifungal resistance.

Future Perspectives

During the development of this research several questions were raised and should be addressed in future studies:

- The contribution of other concomitant medications, like antibacterial drugs, albumin infusion and other catecholamines on the development of antifungal resistance or tolerance;
- The knowledge of the underlying mechanisms of induction of drug resistance by non-antifungal drugs like catecholamines, addressing in particular the identification and characterization of other possible receptors that might be involved, as well as the downstream events implicated;
- Since ibuprofen can act at the transcriptional level, the search for mutations in the transcription factors that regulate azole resistance is mandatory. The effect of ibuprofen in the calcineurin pathway will be studied, aiming also to assess its influence in the dynamics of the induction of antifungal resistance, eventually preventing its emergence;
- The gene *AOX* should deserve further investigation; it may help to further decode the *C. krusei* genome.

CHAPTER V

References

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CHAPTER VI

Summary - Resumo

Summary

Candida represents the most frequent isolated yeast from fungaemia patients. The economic cost of bloodstream fungal infections and its associated mortality, especially in debilitated patients, remains unacceptably high. Fungi are highly adaptable microorganisms, developing resistance to antifungal drugs whenever under its pressure. There are multiple mechanisms of antifungal resistance: formation of biofilms which diminish the accessibility of the antifungal, selection of spontaneous mutations that increase expression or decreased susceptibility of the target, altered chromosome stoichiometry and overexpression of multidrug efflux pumps. Besides the ability to develop antifungal resistance, the facility to adapt to different environmental niches in the human body contributes to clinical resistance and a poor outcome.

The research plan of this work addressed the source of candidaemia and the factors that contribute to antifungal resistance or tolerance, both *in vitro* and *in vivo* by pathogenic *Candida* spp. Also, the assessment of *in vivo* effect of the non antifungal drug ibuprofen that was previously shown to be able to revert the resistant phenotype *in vitro*, was also pursued.

The genetic relatedness of *C. albicans* isolates from blood and other biological products from fungaemia patients revealed that distinct patients were infected by the same strain at different time periods, and an increase in antifungal resistance was observed over time for some of these strains. These results were suggestive of an hospital-acquired exogenous nature of the infection, by hospital endemic strains.

Fungi represent well characterized eukaryotic model systems for scientific and biomedical research. The ability to develop resistance mechanisms *in vivo* is well documented among patients under azole antifungal therapy. Echinocandins brought new hope for treatment of candidaemia and nowadays are considered first line drugs. However, even with the newly available anidulafungin, structural alterations in the HS1 of *FKS*2 molecule due to point mutations can lead to a dramatic decrease in drug efficiency *in vivo*, in a patient with *C. glabrata.* Even though only few reports described echinocandin decreased susceptibility *in*

vivo, ours was the first one addressing anidulafungin. In fact, *Candida* may exploit compensatory cell wall chitin production following exposure to echinocandins in order to overcome the decrease of β-glucan in the cell wall. This paradoxical effect can now be predicted with a new reliable flow cytometric protocol using calcofluor white staining.

Metabolic adaptability mechanisms, including alterations in the respiratory mitochondrial pathway, can also modulate antifungal resistance or tolerance to stress. Although unrelated to intrinsic resistance to fluconazole, the hereby described alternative respiratory pathway in *C. krusei* protects the yeast from oxidative stress.

Critical care patients are often administrated multiple medications including life support therapy. This represents risk factors for increased clinical resistance and consequently to a poor outcome. Propofol lipidic infusion, often used for sedation in mechanically ventilated patients, reduced the access and/or permeabilization of antifungal agents into the fungal cell. Vasoactive amines like adrenaline are lifesaving medications, often administered in ICU. In humans, the adrenergic receptors, a class of G protein-coupled receptors (GPCRs), represent catecholamine targets. Several studies have described the existence of G-protein coupled receptors in yeasts, showing an identical GTP binding site to human GPCRs. Considering the many homologies that have already been described between yeasts and human cells, we raised the hypothesis that in *Candida albicans* adrenaline could act through similar adrenergic receptors and influence antifungal resistance. Such studies showed that adrenaline can improve the fitness of fungal cells by stimulating energy production and fungal growth, thus allowing overcoming the stress caused by the antifungal drug by increasing efflux activity.

The knowledge of the antifungal resistance mechanisms can allow the design of alternative therapeutically options in order to modulate or revert the resistance. In a murine model of *C. albicans* systemic infection, ibuprofen acted synergistically with fluconazole, by reducing drastically the fungal burden in mice infected with a resistant *C. albicans* strain. By allying anti-inflammatory and analgesic properties, ibuprofen in combination with fluconazole might play a relevant role in a future therapeutic strategy for severe fungal infections.

This research results in a more comprehensive understanding of the multiple mechanisms that can influence antifungal resistance or tolerance. The deliverables of our studies include a better awareness of the efforts that should be made for the implementation of routine antifungal susceptibility testing and provide new hope for the prevention of azole antifungal resistance.

Sumário

Candida é a levedura mais frequentemente isolada em doentes com fungemia. A mortalidade associada às infeções fúngicas, especialmente em pacientes debilitados, permanece inaceitavelmente alta. Os fungos são microrganismos altamente adaptáveis, capazes de desenvolver resistência a fármacos antifúngicos sempre que estejam sob a sua pressão de seleção. Existem vários mecanismos de resistência antifúngica: formação de biofilmes que diminuem o acesso do antifúngico, aumento da expressão ou diminuição da suscetibilidade do alvo, alteração da estequiometria cromossómica ou sobre-expressão de transportadores de efluxo. Para além desta capacidade para desenvolver resistência antifúngica, a *Candida* tem muita facilidade para se adaptar a diferentes nichos ambientais no corpo humano, contribui para a resistência clínica e o mau prognóstico.

Este trabalho procurou por um lado esclarecer a origem das estirpes que provocam candidemia e, por outro lado, caracterizar os fatores que podem contribuir para a resistência ou tolerância *in vitro* e *in vivo* de espécies patogénicas de *Candida* aos antifúngicos. Para além disso, avaliou-se o efeito *in vivo* de um fármaco não antifúngico como o ibuprofeno, para o qual se demonstrou que ser capaz de reverter o fenótipo de resistência *in vitro* ao fluconazole.

A identidade genética de *C. albicans* isolada em diferentes doentes a partir de sangue e de outros produtos biológicos de doentes com fungemia revelaram que foram infetados pela mesma estirpe em períodos de tempo distintos, observando-se também um aumento da resistência antifúngica em algumas estirpes. Estes resultados foram sugestivos de uma infeção de natureza exógena adquirida em ambiente hospitalar, por estirpes provavelmente endémicas no hospital.

O desenvolvimento *in vivo* de resistência da *Candida* aos antifúngicos está bem documentado em doentes sob tratamento com azoles antifúngicos. As equinocandinas conduziram a uma nova esperança para o tratamento da candidemia e hoje em dia são considerados fármacos de primeira linha. Contudo, mesmo com a recentemente disponível anidulafungina, podem ocorrer alterações estruturais no HS1 da molécula *FKS2*, devido a

mutações pontuais, que podem levar a uma diminuição abrupta na eficiência do fármaco *in vivo*. Estão descritos casos clínicos de diminuição da suscetibilidade das equinocandinas *in vivo.* No entanto, não estava ainda descrita essa diminuição de suscetibilidade para a anidulafungina. O caso clínico relatado neste trabalho mostra pela primeira vez essa redução da suscetibilidade *in vivo*. Após a exposição às equinocandinas, a *Candida* produz mais quitina, que compensa a diminuição de β-glucano na parede celular. Este efeito paradoxal pode agora ser previsto com um protocolo de citometria de fluxo fiável, usando o calcofluor como marcador.

Os mecanismos de adaptação metabólica, incluindo alterações na via respiratória mitocondrial, podem também modular a resistência antifúngica ou atolerância ao stress. Embora não relacionado com a resistência intrínseca ao fluconazole, a cadeia respiratória alternativa da *C. krusei* protege a levedura do stress oxidativo.

Aos doentes em estado crítico são normalmente administradas variadas medicações de suporte de vida, que podem ser um fator de risco para o desenvolvimento de resistência clínica aos antifúngicos e consequentemente para um mau prognóstico. A infusão lipídica de propofol, muitas vezes usada para sedação em doentes ventilados, reduz o acesso e/ou permeabilização dos agentes antifúngicos à célula. As aminas vasoativas, tal como a adrenalina, são medicamentos frequentemente administrados nas Unidades de Cuidados Intensivos para estabilizar a função cardiocirculatória. Nos seres humanos, as catecolaminas atuam em recetores adrenérgicos que pertencem à família dos recetores articulados a proteínas G (GPCR). Os fungos são um modelo muito bem caracterizado para a investigação científica e biomédica. Está descrita a existência destes recetores GPCR em leveduras com um local de ligação ao GTP idêntico ao dos GPCR dos seres humanos. Considerando as várias homologias entre leveduras e células humanas, postulámos a hipótese de que a adrenalina poderia atuar na *C. albicans* ligando-se a GPCR semelhantes aos recetores adrenérgicos humanos, influenciando assim a resistência aos antifúngicos. Estes estudos revelaram que a adrenalina aumenta a produção de energia, o crescimento fúngico, e a atividade dos transportadores de efluxo de antifúngicos azólicos.

O conhecimento dos mecanismos de resistência antifúngica permitem desenhar alternativas terapêuticas de forma a reverter a resistência. Num modelo animal de infeção sistémica por *C. albicans*, o ibuprofeno atuou de forma sinérgica com o fluconazole, reduzindo drasticamente a carga fúngica nos ratos infetados com uma estirpe resistente de *C. albicans*. O ibuprofeno, bem conhecido como medicamento analgésico e anti-inflamatório, poderia ainda desempenhar um papel relevante numa futura estratégia terapêutica para reduzir a resistência ao fluconazole em doentes com infeções fúngicas graves.

Esta dissertação resultou numa compreensão mais abrangente dos múltiplos mecanismos que podem influenciar a resistência ou tolerância aos antifúngicos. Os resultados dos nossos estudos incluem um melhor conhecimento dos esforços que devem ser tomados para a aplicação na rotina dos testes de suscetibilidade aos antifúngicos e para dar uma nova esperança na prevenção da resistência aos azoles antifúngicos.

CHAPTER VII

Publications

RESEARCH ARTICLE

An alternative respiratory pathway on *Candida krusei*: implications on susceptibility profile and oxidative stress

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Candida krusei; alternative respiratory pathway; antifungal resistance; oxidative stress; alternative oxidase.

Abstract

Our aim was to detect the presence of an alternative oxidase (AOX) in *Candida krusei* clinical strains and its influence on fluconazole susceptibility and in reactive oxygen species (ROS) production. *Candida krusei* clinical isolates were tested to evaluate the presence of AOX. *Debaromyces hansenii* 2968 (AOX positive) and *Saccharomyces cerevisiae* BY4742 (AOX negative) were used as control strains. Measurements of oxygen consumption were performed in the presence of 1 mM KCN, an inhibitor of the classical respiratory chain, and 5 mM salicylhydroxamic acid (SHAM). AOX expression was monitored by Western blotting using an AOX monoclonal antibody. Interactions between fluconazole and SHAM were performed using checkerboard assay. ROS production was evaluated in the presence of SHAM plus fluconazole, H_2O_2, menadione, or plumbagin. AOX was present in all *C. krusei* tested. The combination of fluconazole with SHAM resulted in an indifferent effect. In the presence of SHAM, the treatment with ROS inductors or fluconazole increased ROS production, except in the AOX-negative strain. An alternative respiratory pathway resistant to cyanide is described for the first time as a characteristic of *C. krusei* species. This AOX is unrelated to fluconazole resistance; however, it protects *C. krusei* from oxidative stress.

Introduction

Over the past few decades, authors have documented increases in the rate of candidemia by non-*albicans* species of *Candida*, such as *Candida krusei*, especially in critically ill and immunocompromised patients with hematologic malignancies (Hachem *et al.*, 2008).

Fluconazole is often used in the prophylaxis and treatment of candidemia and is the first-line therapy for this condition (Pappas *et al.*, 2009). Among non-*albicans* species, *C. krusei* is the only species that is predictably fluconazole resistant (Oxman *et al.*, 2010). Antifungal drug tolerance can also be modulated by metabolic adaptability mechanisms, including alterations in the respiratory mitochondrial pathway (Chamilos *et al.*, 2006; Brun *et al.*, 2003). This fact had been previously assessed in *C. glabrata*, *C. albicans*, and *C. parapsilosis* regarding its influence on fluconazole and caspofungin (Brun *et al.*, 2003; Chamilos *et al.*, 2006; Yan *et al.*, 2009).

In eukaryotic organisms, the energy necessary for growth, development, reproduction, and stress response is acquired through the ATP synthesized during mitochondrial respiration, where cytochrome *c* oxidase acts as a terminal oxidase in the reception of electrons and converting oxygen into water. Within the mitochondrial respiratory chain, another route mediated by the alternative oxidase (AOX; a mitochondrial enzyme) can be found in plants, in certain protozoa and fungi (Moore & Siedow, 1991; Helmerhorst *et al.*, 2002, 2005; Veiga *et al.*, 2003a, b). This AOX is insensitive to cytochrome pathway inhibitors, such as antimicin A or cyanide, but is specifically inhibited by salicylhydroxamic acid (SHAM) and confers a cyanide-resistant respiration through an alternative respiratory pathway (ARP) to such organisms (Moore

& Siedow, 1991). The AOX is located on the matrix side of the inner mitochondrial membrane and plays an important role in susceptibility to azole antifungals in *C. albicans* (Yan *et al.*, 2009). The alternative respiratory chain can be activated by stress situations like the presence of antifungals or oxidative inductors, thus leading to drug tolerance and to the reduction in generation of intracellular reactive oxygen species (ROS; Cannon *et al.*, 2007).

In an attempt to explain the contribution of mitochondrial respiration in the intrinsic fluconazole resistance displayed by *C. krusei*, we assessed the existence of an ARP and its influence upon fluconazole resistance and tolerance to oxidative stress in *C. krusei*.

Materials and methods

Drugs and chemicals

Fluconazole was obtained from Pfizer (Groton, CT), and stock solutions were prepared according to CLSI M27 A3 protocol and maintained at −70 °C until use (CLSI, 2008). Stock solutions of SHAM (200 mM; Sigma-Aldrich, Germany) and potassium cyanide (KCN, 1 M; Sigma) were prepared in DMSO (Sigma) and distilled water, respectively. Hydrogen peroxide (H_2O_2; 30% v/v) was obtained from Merck. Plumbagin and menadione were obtained from Sigma, and stock solutions (100 mM) were prepared in 95% ethanol. Dihydrorhodamine 123 (DHR123) was obtained from Molecular Probes (Eugene, OR).

Strains and culture conditions

A total of 25 *C. krusei* (nine from respiratory secretions, one from blood, 11 vaginal, three fecal, and the type strain ATCC 6258 from the American Type Culture Collection), previously identified using VITEK II system (BioMérieux, Paris, France), all resistant to fluconazole were used in this study. *Candida albicans* SC5314 strain was used as positive control for the presence of AOX-specific protein. The strains were grown in Sabouraud dextrose broth (Difco) at 30 °C, in an orbital shaker at 150 r.p.m. until late exponential growth phase (OD_{640} 1.5). *Debaromyces hansenii* IGC2968 (LGC Standards S.L. U., Barcelona, Spain) was used as the positive control for the presence of an ARP, assessed by oxygen consumption. *Saccharomyces cerevisiae* BY4742 (EUROSCARF, Frankfurt, Germany) was used throughout the study as the negative control for the presence of the AOX assessed by oxygen consumption, immunoblotting, and ROS production. These control strains were grown in a mineral medium with vitamins and 2% (w/v) glucose until stationary growth phase (OD_{640} 2.5 and 3, respectively; Veiga *et al.*, 2003a, b).

Alternative respiratory pathway

Late exponential phase cultures were centrifuged (18 000 ***g***) for 4 min at 4 °C and washed twice with cold sterile water. An small amount (1.5 g wet weight) of pellet was resuspended in 100 mL of 50 mM phosphate-buffered saline, pH 6.0 with 0.1% cicloheximide (Sigma, St. Louis, MO), and 6 mL of suspension was incubated in a small reactor at 28 °C. The O_2 consumption was continuously measured with a Clark-type electrode YSI model 5775 (YSI Incorporated, Yellow Springs, OH) after the addition of 200 μL of 1.55 M glucose (Difco Laboratories, Detroit, MI), 3.2 mM of KCN, and/or 3.2 mM of SHAM (Sigma-Aldrich). The presence of an AOX was considered whenever the oxygen consumption pattern was resistant to KCN, but sensitive to SHAM.

Checkerboard microdilution assay

Checkerboard assays were performed with all *C. krusei* strains, in the presence of fluconazole and SHAM, using the protocol described in the Clinical Microbiology Procedures Handbook (Moody, 1991). The concentration range used was 0.125–64 μg fluconazole mL^{-1} and 0.6–20 mM for SHAM. The minimal inhibitory concentrations (MIC) of each compound were determined according to CLSI M27 A3 and S3 protocol in RPMI 1640 (Sigma; CLSI, 2008). Fractional inhibitory concentration of fluconazole (FIC_A) was calculated as the MIC of fluconazole in combination/MIC of fluconazole alone, and FIC_B is the MIC of SHAM in combination/MIC of SHAM alone. The fractional inhibitory index (FIX) was calculated as follows: $FIX = FIC_A + FIC_B$. The interpretation of FIX was as recommended: ≤ 0.5, synergistic effect; > 0.5 to < 4.0, no interaction; ≥ 4.0, antagonistic effect (Moody, 1991).

Assessment of intracellular ROS accumulation

Intracellular ROS accumulation was estimated to study the influence of an AOX upon *C. krusei* oxidative stress tolerance. After being washed and resuspended in PBS, 10^7 cells mL^{-1} were treated with ROS inductors such as H_2O_2 (0.4 mM), menadione (0.5 mM), and plumbagin (0.003 mM) for 15, 30, and 60 min, at 35 °C under 150 r.p.m. Yeast cells were also treated with fluconazole and voriconazole at MIC concentrations for 1 h. All the treatments were repeated following the preincubation of yeast cells with 3.2 mM of SHAM, an inhibitor of the AOX, during 30 min. Control cells were treated with a

DMSO concentration similar to that used in cell samples pretreated with SHAM. The cells were then collected by centrifugation and resuspended in PBS. Free intracellular ROS were detected with 15 μg dihydrorhodamine 123 mL^{-1} (Molecular Probes; Almeida *et al.*, 2007; Mesquita *et al.*, 2010). Cells were incubated during 90 min at 30 °C in the dark, washed in PBS, and 5 μL was placed in a glass slide and overlapped with vectashield fluorescence mounting media (Vector Laboratories, Peterborough, UK). The stained suspensions were visualized under epifluorescence microscopy (40×; Olympus BX61). In each condition, a minimum of 500 cells from three different replicates were counted, and the percentage of stained cells (cells displaying ROS) was determined.

Chronological life span

Overnight cultures of *C. krusei* strains were incubated in YPD broth until stationary phase (day 0 of chronological life span; Mesquita *et al.*, 2010). Cultures were treated without (control) and with 3.2 mM SHAM, 0.25 mM, and 0.5 mM of menadione alone, and in combination with 3.2 mM of SHAM. Survival was assessed daily by counting colony-forming units (CFUs) from cultures aliquots in YPD agar plates beginning at day 0 (when viability was considered to be 100%).

Preparation of mitochondrial extracts

Mitochondrial extracts were isolated from *S. cerevisiae*, *Candida albicans* SC5314, and *C. krusei*. Cells were grown to stationary phase, harvested, and cell wall was digested with zymolyase buffer [2 M sorbitol-D, phosphate buffer 1 M (pH 7.5), zymolyase 20 000 U, 125 mM β-mercaptoethanol, 0.5 M EDTA] at 30°C for 1 h. Protoplasts were disrupted with lysis buffer [sorbitol-D 0.5 M, Tris 20 mM, EDTA 1 mM, and 2.85 mM phenylmethanesulphonyl fluoride (PMSF)] using a Potter homogenizator. Mitochondrial extracts were separated, washed by high-speed centrifugation at 16 420 ***g*** for 15 min at 4°C (Beckman Coulter, JA-25.50 Rotor), and resuspended in sorbitol buffer (0.5 sorbitol-D, 5 mM EDTA, 50 mM Tris). Protein concentration was determined by Bradford method (Kruger, 1994), and protein aliquots of mitochondrial extracts (40 μg) were stored at −20°C.

Immunoblotting

Mitochondrial protein extracts were resolved on a 12% SDS gel and transferred to a nitrocellulose membrane. The membrane was blocked with phosphate-buffered saline (TBS) with 0.1% Tween 20 (TBST) containing 5% skim milk, followed by incubation with antibodies against AOX proteins (1 : 100) of *Sauromatum guttatum* (Agrisera AB, Vännas, Sweden) in TBST containing 1% skim milk. After washing with TBS, the membranes were incubated with the respective secondary antibody, HRP-conjugated anti-mouse IgG at a dilution of 1 : 5000, and detected by enhanced chemiluminescence.

Statistical analysis

All experiments were performed in triplicate. Mean values were compared using Student's *t*-test whenever indicated. A P value < 0.05 was considered statistically significant.

Results

Presence of a cyanide-resistant respiration pathway in *C. krusei*

To investigate whether an ARP was present in clinical isolates of *C. krusei*, measurements of O_2 consumption were performed in the presence of 3.2 mM of cyanide, an inhibitor of the classical respiratory chain (by inhibiting the cytochrome *c* oxidase complex), and SHAM, an inhibitor of the ARP (by inhibiting the AOX). In *C. krusei* strains, O_2 consumption stopped only after the addition of SHAM (Fig. 1a, representative example). This observation was made for all the 24 *C. krusei* clinical isolates, with ATCC 6258 type strain and with the positive AOX control strain *Debaromyces hansenii* 2968 (Fig. 1b). In *Saccharomyces cerevisiae* BY4742, the negative AOX control strain, the oxygen consumption stopped soon after the addition of cyanide (Fig. 1c). The results indicate that *C. krusei* cells have a cyanide-resistant respiration pathway promoted by the presence of an AOX.

AOX expression

Mitochondrial preparations were immunoblotted with a monoclonal antibody raised against *S. guttatum* AOX, but also recognize *C. albicans* AOX (Huh & Kang, 1999). In all *C. krusei* clinical isolates, mitochondrial AOX was clearly detectable as well as in *C. albicans* SC5314 strain (Fig. 2). Mitochondria isolated from the negative control strain, *S. cerevisiae* BY4742, showed no reactivity with AOX antiserum (Fig. 2).

Fluconazole–SHAM combination did not influence fluconazole activity

To study the influence of the ARP upon fluconazole resistance by *C. krusei*, we explored the *in vitro* combination

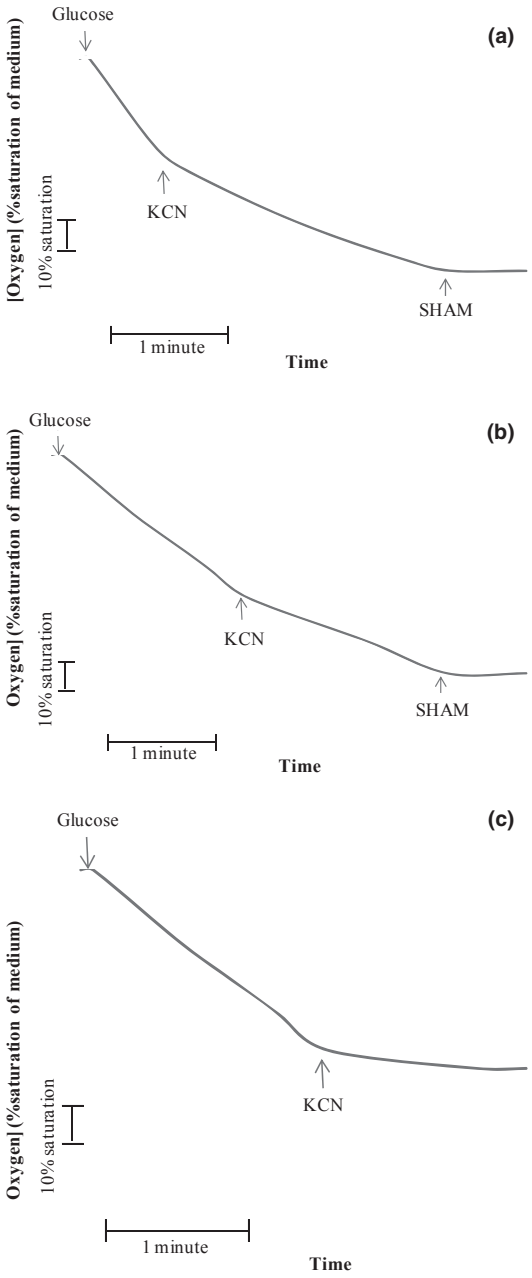

Fig. 1. Effect of KCN and SHAM upon oxygen consumption by *Candida krusei* clinical strain (representative example) (a), *Debaryomyces hansenii* (AOX positive control strain) (b), and *Saccharomyces cerevisiae* (AOX negative control strain) (c). Oxygen consumption was measured with an oxygen electrode at 28 °C. Where indicated (arrows), glucose (0.051 mM), KCN (3.2 mM), and SHAM (3.2 mM) were added.

of fluconazole and SHAM using the checkerboard methodology. SHAM produced no impairment of antifungal activity even at a concentration of 10 mM. The addition of SHAM did not change MIC values for fluconazole, resulting in an indifferent effect in all *C. krusei* isolates.

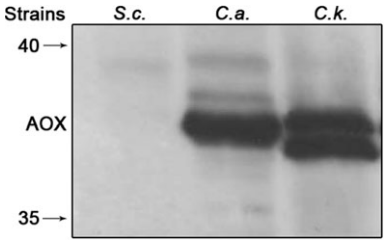

Fig. 2. Representative example of the presence of an AOX in *Candida krusei*. Immunoblot analysis of AOX levels in 40 μg of mitochondrial extracts of *Saccharomyces cerevisiae* (*S.c.*), *Candida albicans* SC5314 (*C.a.*), and *Candida krusei* (*C.k.*).

The presence of an alternative respiratory pathway in *C. krusei* relates to reduced ROS accumulation

To assess the influence of the ARP upon oxidative stress response, we measured intracellular ROS accumulation with and without SHAM, using DHR123. This fluorochrome enters the yeast cell as a freely permeable dye, which is converted to rhodamine 123 and subsequently localized in the mitochondria. The conversion from the nonfluorescent to the fluorescent molecule is entirely dependent upon the presence of oxidation products. After treatment with the ROS-inducing agents, H_2O_2, plumbagin, menadione, and with azoles, the percentage of stained cells (cells with ROS accumulation) was calculated and compared with values displayed by nontreated cells, in the presence and absence of SHAM (3.2 mM). No significant differences regarding ROS accumulation were observed with the AOX-negative strain, *S. cerevisiae* BY4742, in the presence or in the absence of SHAM (Fig. 3). In contrast, after treatment with ROS-inducing agents, *C. krusei* cells treated with SHAM produced significantly ($P < 0.001$) more endogenous ROS than the cells with the unblocked ARP (without SHAM; Fig. 3). Significant differences in ROS production ($P = 0.008$) were also accomplished with fluconazole (Fig. 3). All *C. krusei*-tested strains displayed similar results.

Chronological life span decreased in *C. krusei* strains incubated with menadione when compared with untreated cultures (Fig. 4). The viability of *C. krusei* strains reduced significantly after 3 days of incubating the cultures with menadione plus SHAM (Fig. 4).

Discussion

Several authors have stressed the relevance of the mitochondrial respiration and its influence upon metabolic behavior, stress environment adaptability, and antifungal drug tolerance (Brun *et al.*, 2003; Chamilos *et al.*, 2006; Yan *et al.*, 2009; Li *et al.*, 2011). A mitochondrial ARP

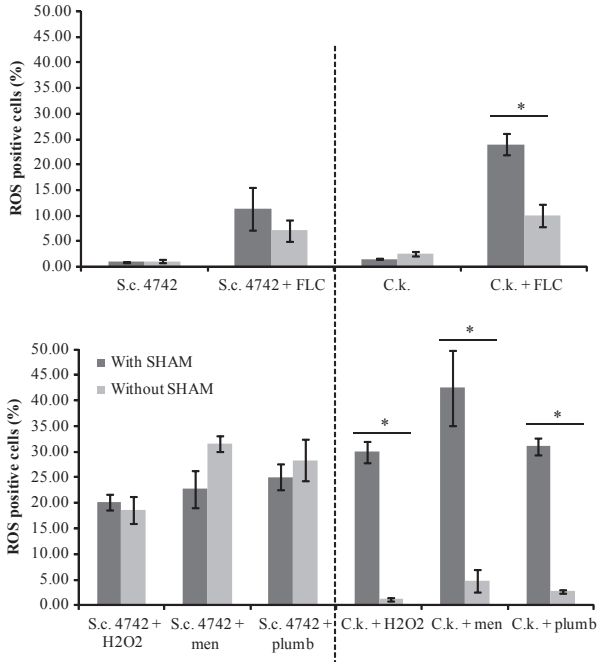

Fig. 3. Effect of fluconazole (FLC) and oxidative inductors (menadione – Men; plumbagin – Plumb; hydrogen peroxide – H_2O_2) upon intracellular ROS accumulation by a clinical *Candida krusei* (*C.k.*) strain (representative example) and a negative control strain *Saccharomyces cerevisiae* (*S.c.*), with or without the addition of SHAM. ROS accumulation was calculated and expressed as the percentage of DHR123-stained cells (*$P < 0.05$).

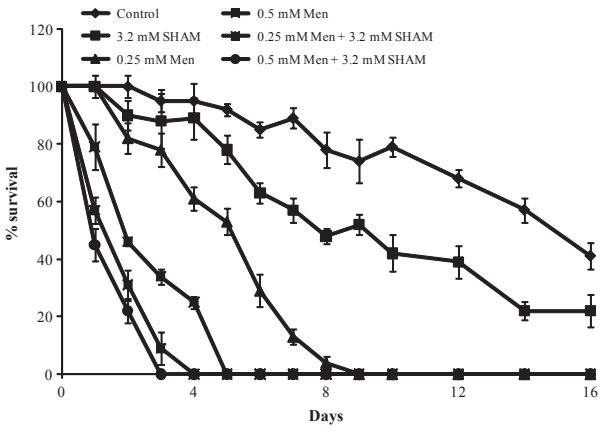

Fig. 4. Chronological life span of a *Candida krusei* strain. Strains were incubated without (control) and with 3.2 mM SHAM, 0.25 mM, and 0.5 mM of menadione (Men) alone and in combination with 3.2 mM of SHAM. Survival was assessed daily by counting colony-forming units (CFUs) from cultures aliquots in YPD agar plates beginning at day 0 (when viability was considered to be 100%).

(cyanine-resistant) occurs in all higher plants, in many fungi, and in some protozoa (Kirimura *et al.*, 1999; Johnson *et al.*, 2003). Such a pathway uses electrons from the ubiquinol pool to reduce oxygen to water, bypassing the complex III and the cytochrome oxidase complex, two sites of energy conservation in the main respiratory chain. An AOX, sensitive to SHAM and resistant to cyanide, is responsible for this alternative pathway (McIntosh, 1994). The cyanide-resistant respiration has been previously described in *C. albicans* and *C. parapsilosis* (Helmerhorst *et al.*, 2002; Chamilos *et al.*, 2006; Yan *et al.*, 2009). In this study, we described for the first time the presence of an ARP mediated by an AOX in *C. krusei*.

The elucidation of the oxidative stress responses in yeast has considerable clinical interest, as it is involved in invasion and colonization of host tissues by yeast pathogens as well as during the defensive mechanisms triggered by phagocytes. All aerobic organisms inevitably generate a range of ROS, including superoxide anion, hydrogen peroxide, and hydroxyl radical during oxygen metabolism. If not quickly and effectively eliminated from the cells, ROS will trigger a large number of oxidative reactions in cellular systems that possibly lead to cell death (Raha & Robinson, 2000). In the course of an *in vivo* infection, the formation of ROS and other oxidants radicals by phagocytes plays a crucial role in the intracellular destruction of the pathogen (Murphy, 1991). ROS attack almost all essential cell components, including DNA, proteins, and lipids (Moradas-Ferreira & Costa, 2000). In a recent study, *C. krusei* appears to be resistant to ROS and to possess a potent antioxidant system enabling deep systemic infections (Abegg *et al.*, 2010). We decided to evaluate the difference regarding oxidative stress response before and after the blockade of the ARP. It has been previously described that in *C. albicans*, fluconazole is able to induce the production and accumulation of ROS (Kobayashi *et al.*, 2002). In our study, we showed that fluconazole induced a low percentage of ROS formation by *C. krusei* cells. These results may suggest that the fungistatic mechanism of this azole is not based upon ROS formation. However, when the AOX was inhibited by SHAM, an increase in the intracellular ROS levels was evident. Attending to these facts, we can conclude that AOX activity allows the yeast cells to reduce ROS accumulation when challenged by antifungals like fluconazole, leading to drug tolerance, like in *C. albicans* (Yan *et al.*, 2009). According to several authors, the AOX has a metabolic and antioxidant role, and its presence may be considered a potential virulence attribute of pathogenic fungi (Vanlerberghe *et al.*, 2002; Missall *et al.*, 2004; Yan *et al.*, 2009). The importance of AOX activity upon resistance to oxidative stress was evident when the oxidative stress inductors, H_2O_2, menadione, and plumbagin, were assayed. After treatment with such compounds, ROS accumulation was low. However, the scenario changed significantly when the AOX activity was blocked by

SHAM. To confirm our hypothesis that the presence of an ARP could protect *C. krusei* from oxidative stress, we assessed ROS accumulation in the presence of fluconazole and the other oxidative stress inductors by the negative control strain, *S. cerevisiae* (AOX-). The results obtained regarding ROS accumulation, in the presence or absence of SHAM, were not significantly different. When testing *C. albicans* AOX mutant strains, Yan and coworkers also obtained no significant differences in the amount of ROS generation (Yan *et al.*, 2009). Regarding the chronological life span assays, we could conclude that the decreased viability of *C. krusei* strains in the presence of menadione and with the AOX blocked is associated with an increased level of cell ROS.

Our results showed clearly that the inhibition of the expression of the AOX was associated with intracellular ROS accumulation, revealing the effect of the ARP on oxidative damage. Although fluconazole resistance was unrelated to the presence of the ARP, we can consider that it confers antifungal tolerance, which may give yeast cells enough time to develop long-term genetically stable resistance mechanisms (Cannon *et al.*, 2009).

The ARP is a potential target that should be taken into account when considering the development of new therapeutic strategies in the case of *C. krusei* infections. Attending to its selective effect, SHAM should be used in combination with azoles, to reduce resistance because of oxidative stress and consequently the virulence of *C. krusei*.

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FKS2 Mutations Associated with Decreased Echinocandin Susceptibility of *Candida glabrata* following Anidulafungin Therapy▽

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This is the first case report of *Candida glabrata*-disseminated candidiasis describing the acquisition of echinocandin resistance following anidulafungin treatment. The initial isolates recovered were susceptible to echinocandins. However, during 27 days of anidulafungin treatment, two resistant strains were isolated (from the blood and peritoneal fluid). The resistant peritoneal fluid isolate exhibited a Ser663Pro mutation in position 1987 of *FKS2* HS1 (hot spot 1), whereas the resistant blood isolate displayed a phenylalanine deletion (Phe659).

Candida glabrata infections represent a serious clinical problem due to patient comorbidities, the inherent high mortality rate, and the predisposition to rapidly develop azole resistance (6, 11, 20). Therefore, efforts were made to develop new therapeutic alternatives. A new class of antifungal agents has arisen, including caspofungin (CSF), micafungin (MCF), and more recently, anidulafungin (ANF), which inhibit β (1,3)-glucan synthase activity. This enzyme, including the regulatory subunit Rho-1p, is responsible for producing β (1,3)-glucan, a key component of the fungal cell wall, and is encoded by *FKS* genes.

Due to their excellent clinical effectiveness and safety profile, these antifungals became the first-line therapy in many hospitals for the treatment of invasive candidiasis (17, 18, 26). Despite the wide use of CSF, reports describing primary or secondary echinocandin resistance in candidiasis are rare. Reduced susceptibility to echinocandins has been linked to mutations in hot spot regions of *FKS* genes (19). We report for the first time the *in vivo* acquisition of echinocandin resistance following ANF therapy in a patient with *C. glabrata* invasive candidiasis.

A 71-year-old female patient was admitted to the intensive care unit (ICU) with a diagnosis of acute pancreatitis that developed after a laparoscopic cholecystectomy. The patient's medical history included hypertension and dyslipidemia. An exploratory laparoscopy was performed on the second day after admission to the ICU. The surgical findings were peritoneal necrosis, necroses of the ileum and jejunum, and pancreatic necrosis. During the hospital stay, several antibacterial drugs were administered to the patient, namely tazobactam, piperacillin, meropenem, tigecycline, and vancomycin. She had no previous antifungal exposure. Her medical condition was further complicated by multiple *Candida* infections, including disseminated candidiasis and central venous catheter-related infections. Starting on day 6, she received a 200-mg load of ANF, followed by 100 mg ANF/day for 27 days. Cultures of multiple sites, including blood, exudates, central venous catheter (CVC), and urine, were positive for *C. albicans* and *C. glabrata.* While *C. albicans* fungemia cleared following 24 h of ANF therapy, *C. glabrata* persisted. On day 17, the patient received noradrenaline support. Subsequently, she was submitted to a second exploratory laparoscopic surgery on day 30. The procedure revealed extensive cytosteatonecrosis. Amphotericin B was then initiated, and 2 days later, the patient developed multiorgan failure. The patient died 34 days after admission. No autopsy was performed.

Twenty-one isolates were recovered from the patient's blood and other biological samples throughout ANF treatment (Fig. 1). The assessment of the MICs of azoles, amphotericin B, and echinocandins for all clinical isolates was performed in RPMI 1640 (Sigma), according to the CLSI M27-A3 protocol (4, 5).

From the set of *C. glabrata* isolates, two isolates were resistant to echinocandins, one recovered from the blood (isolate 18-1) and the other from the peritoneal fluid (isolate 30-2) (isolated 18 and 30 days after ICU admission, respectively) (Fig. 1 and Table 1). The acquisition of ANF resistance was associated with an increase in the CSF and MCF MIC values, suggesting the development of cross-resistance among these three compounds. Although MIC values are usually considered predictors of clinical response to antimicrobial therapy in invasive fungal infections, no strong correlation has yet been

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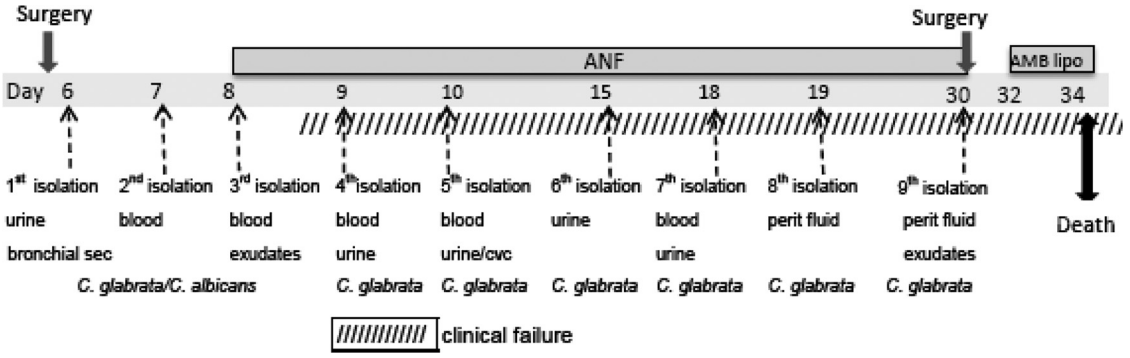

FIG. 1. Time line of antifungal therapy administered to the patient. The number of days after admission and fungal strains isolated from biological samples are also shown. ANF, anidulafungin; AMB lipo, amphotericin B lipid complex.

found between *in vitro* susceptibility results and clinical outcome (6, 12, 13, 15, 16, 21, 22).

In the clinical case described herein, a correlation between the *in vitro* results and the *in vivo* lack of efficacy of the drug was found. The MIC values obtained (4 μg/ml) from the blood (strain 18-1) and peritoneal fluid (strain 30-2) isolates were above the susceptibility breakpoint.

Random amplification of polymorphic DNA (RAPD) was carried out using isolates obtained from different biological samples to determine their isogenicity. Using the primers OPE-18 (5′ GGACTGCAGA 3′) and OPA-18 (5′ AGCTGA CCGT 3′) (2), identical band profiles were displayed by all isolates, with the exception of strain 30-2, which was recovered from the peritoneal fluid prior to patient death (Fig. 2). Considering the surgical interventions, the evidence of CVC infection, and the long stay in the ICU (34 days), where nosocomial infections are likely to occur, it is plausible that this strain had an exogenous source.

Mutations in *FKS* genes have been shown to be responsible for echinocandin resistance during caspofungin treatment, namely of *C. glabrata* (3, 9, 10, 14, 24). To determine whether ANF resistance was associated with mutations in the target genes, genomic DNA of *C. glabrata* isolates was extracted and, using the hot spot 1 (HS1) regions of the *FKS1* and *FKS2* genes, were amplified with specific primers (*FKS1* HS1F2 [5′-CTTATGTTTGATTTTTGCA-3′]) (8, 24). DNA products were sequenced in an ABI Prism 3130 genetic analyzer (Applied Biosystems). The coding sequences of the *Candida glabrata FKS1* and *FKS2* genes (GenBank accession numbers XM_446406 and XM_448401, respectively) were aligned with those obtained from the clinical isolates. No sequence alterations were observed in HS1 of the *FKS1* gene; however, several point mutations were found in HS1 of the *FKS2* gene, with most of them corresponding to synonymous substitutions that did not result in nucleotide changes. Furthermore, the isolate 30-2, which was recovered following a long period of ANF exposure, displayed a C-T mutation at position 1987. This mutation leads to a replacement of serine 663 by proline in HS1 of *FKS2* (Table 1). In the same HS, a deletion of 3 nucleotides was found in the blood isolate 18-1, which results in the deletion of one of the two consecutive phenylalanines at positions 658 and 659 (Table 1). These findings are consistent with previous reports describing increased resistance to echinocandins associated with mutations, namely, the S663P substitution and Phe659 deletion in the *FKS2* gene (9, 10). The highest frequency of resistance-associated mutations is found within HS1 (1). HS1 is a highly conserved region among the Fks family; hence, the amino acid changes in this region implicate a modification of the echinocandin target and a reduced susceptibility phenotype.

The significant increase in the chitin content following *in vitro* echinocandin exposure has been suggested as an escape or salvage mechanism for echinocandins (7, 23, 25). In order to unveil the role of chitin in the resistance displayed by isolates 18-1 and 30-2, the cell wall chitin content was measured initially and after 30 days of subculturing in drug-free medium. While the resistant phenotype remained, the chitin content decreased (data not shown). Attending to such results, we were compelled to conclude that the deletion and the mutation detected in the *FKS2* gene confer echinocandin resistance to the isolates 18-1 and 30-2, respectively.

Overall, our findings suggest that structural alterations in the

TABLE 1. *In vitro* antifungal susceptibility of *C. glabrata* isolates to CSF, ANF, and MCF[a]

Strain	Isolation (no. of days after admission)	Sample type	MIC_{50} (μg/ml)			*FKS2* HS1 SNP	AA profile
			CSF	ANF	MCF		
10-1	10	Blood	0.125	≤0.06	≤0.06	Positions 1987-1989, TCT	Ser
18-1	18	Blood	>32	4	4	Positions 1977-1979, CTT deletion	ΔPhe659
19-1	19	Peritoneal fluid	≤0.06	≤0.06	≤0.06	Positions 1987-1989, TCT	Ser
30-2	30	Peritoneal fluid	32	4	8	T1987C	Ser663Pro

[a] Determined according to the CLSI M27-A3 protocol (4). MICs were determined by considering a prominent inhibition, corresponding to 50%, as the endpoint. SNP, single nucleotide polymorphism; AA, amino acid.

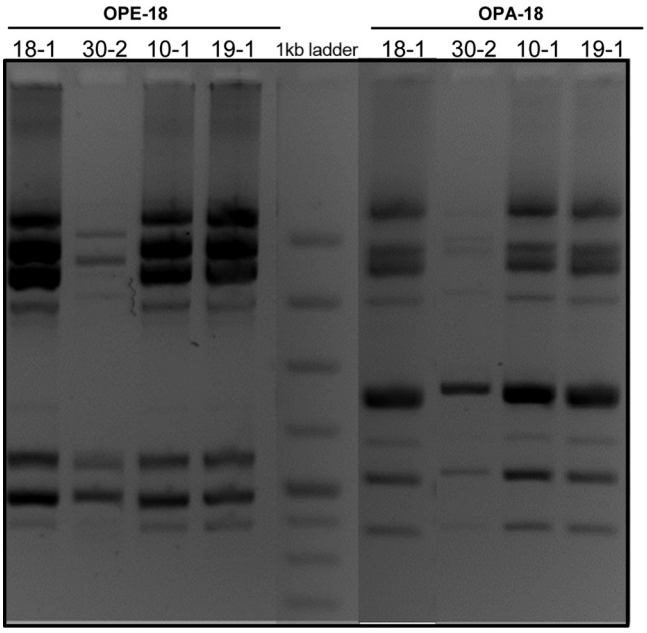

FIG. 2. Random amplification of polymorphic DNA gel patterns of *C. glabrata* isolates 18-1, 30-2, 10-1, and 19-1, resistant and susceptible to echinocandins, obtained with primers OPE-18 and OPA-18.

HS1 of the *FKS2* molecule due to the Ser663Pro substitution and Phe659 deletion lead to a dramatic decrease in echinocandin efficacy. Although just a few cases describing the development of echinocandin resistance have been reported so far, our case report emphasizes the crucial need for antifungal susceptibility surveillance in patients under extended echinocandin therapy.

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Genetic relatedness and antifungal susceptibility profile of *Candida albicans* isolates from fungaemia patients

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A prospective study to assess fungaemia was conducted for 12 months at a Portuguese University Hospital. A total of 35 *Candida albicans* isolates obtained from 12 patients with fungaemia were compared by a multiplex PCR system using four microsatellite loci. Blood isolates were evaluated against concomitant isolates from urine, lower respiratory secretions and central venous catheters, as well as with successive isolates recovered from recurrent episodes of fungaemia. The data analyzed included the department of admission, underlying diseases and antifungal therapy. The susceptibility phenotypes of all isolates to amphotericin B, fluconazole, itraconazole, voriconazole and caspofungin were determined according to the CLSI M27-A3 protocol. We observed a high degree of similarity between successive blood isolates and between blood and concomitant isolates from other sites of the same patient. This is suggestive of the recurrence of fungaemia and was due to the same strain, possibly as a result of the failure of antifungal therapy. The genetic similarity observed between some strains isolated from different patients suggested the likelihood that they were hospital acquired. Distinct patients were infected by the same strain at different time periods, and an increase in antifungal resistance was observed over time for some of these strains. Hospital-acquired exogenous nosocomial infections can be associated with higher risks of antifungal resistance and need to be closely monitored. Particular attention should also be given to endogenous non-blood *Candida* isolates which can be critical in high risk patients, as they often can become invasive in immunodeficient individuals.

Keywords microsatellite, *Candida albicans,* fungaemia, genetic relatedness, antifungals, infection

Introduction

Candida infections have progressively emerged as major nosocomial invasive fungal diseases since the late 1980s, mainly arising from an endogenous source, i.e., either digestive or mucocutaneous. Commensalism, followed by colonization, usually precedes dissemination, most frequently in patients with transient or permanent immunocompromised status, such as transplant recipients, chemotherapy patients, underweight neonates and those infected by the human immunodeficiency virus. Significant morbidity and mortality rates have been associated with *Candida* spp. bloodstream infections [1–3].

Several polymorphic microsatellite loci have been identified in the genome of *C. albicans* near *EF3*, *CDC3* and *HIS3* [4] or inside the coding regions of *ERK1*, *2NF1*, *CCN2*, *CPH2*, and *EFG1* [5]. However the discriminatory power for each locus is relatively low. In order to more rapidly obtain a higher discrimination, simultaneous amplification of sets of microsatellite markers can be performed. A multiplex system with a high discriminatory power was recently described and found to represent an efficient

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molecular tool for the swift and accurate differentiation of *C. albicans* [6,7] isolates.

During a 12-month period (2004) a prospective study addressing fungaemia was conducted at Hospital de São João, a large university hospital located in the Northern region of Portugal [1]. The epidemiological data analyzed included, among others, the department of admission, underlying diseases and antimicrobial therapy. Several yeast isolates from blood cultures were collected and studied, as well as fungi recovered from surveillance cultures or from medical indwelling devices. These included isolates from distinct biological sources of the same patient, such as urine and lower respiratory secretions, and from central venous catheters. All episodes of recurrence were investigated [1].

Our main purpose was to genotype all *C. albicans* isolates by using a multiplex PCR system with four microsatellite loci. Ultimately, we aimed to determine the genetic relatedness between simultaneous and/or recurrent isolates from the same patient, the source of infection, the route of transmission, as well as the possibility of transmission among distinct patients. Additionally, the susceptibility profiles of all isolates were determined and evaluated with respect to antifungal therapy.

Materials and methods

Patients' clinical data

Thirty-five *C. albicans* isolates recovered from the blood and other biological sources were obtained from 12 patients with fungaemia. The data documented included patient gender and age, department and date of admission, concomitant therapy (i.e., immunossuppressors), date and site of fungal isolation, and nosocomial origin of the fungaemia. According to the Centre for Disease Control and Prevention (CDC), nosocomial fungaemia is defined as having occurred whenever at least one fungal positive blood culture is obtained within 48 h of the patient's hospital admission [8]. Antifungal therapy (prophylactic or therapeutic) and clinical outcome (survival/death) were also documented. Outcomes were evaluated 30 days after the first fungaemia episode, with related deaths being defined as those that occurred within 30 days of the first fungal positive blood culture, with no signs of intracerebral or gastrointestinal bleeding or pulmonary embolism. All *Candida* isolates were frozen at –70°C in brain-heart medium with 5% of glycerol (Difco) and subcultured twice on Sabouraud agar (Difco) prior to experimental procedures.

C. albicans *genotyping*

Yeast strains were grown overnight in Sabouraud broth at 30°C. A Zymolyase-based method was used to extract DNA, as previously described [9]. To assess strain relatedness, all isolates were genotyped using a microsatellite multiplex PCR assay with three markers (CAI, CAIII and CAVI) as described by Sampaio *et al*. [7] and a singleplex amplification reaction assay using the microsatellite marker CEF3, according to procedures described by Bretagne *et al*. [4]. Following PCR amplification, a 1–2-µl aliquot of each sample was added to 15 µl of formamide containing 0.4 µl of GeneScan - 500 TAMRA size standards (Applied Biosystems). Amplicons were denatured at 95°C for 5 min and immediately placed on ice. Denatured samples were resolved by capillary electrophoresis in an ABI Prism 310 genetic analyzer (Applied Biosystems). Determination of allele sizes was automatically performed with GeneScan 3.7 analysis software. The alleles were designated according to the number of repeated units for the CAI $[(CAA)_2CTG(CAA)_n]$, CAIII $[(GAA)_n]$ and CAVI $((TAAA)_{n)}$ markers and by the number of nucleotides for the CEF3 $[(TTTC)_n(TTC)_n]$ marker.

Antifungal susceptibility profile

Antifungal susceptibility testing of all isolates was performed according to the CLSI M27 A3 protocol in RPMI 1640 (Sigma) [10,11]. Minimal inhibitory concentration (MIC) for fluconazole, voriconazole, caspofungin and amphotericin B were determined [10,11]. The yeast strains were classified as susceptible (S), susceptible-dose dependent (S-DD) and resistant ® to azoles according to the breakpoints defined by CLSI [10,11]. Although definitive breakpoints have not yet been established for amphotericin B, strains showing MIC $\leq$1 µg/ml were considered susceptible [12]. For caspofungin, strains with an MIC $\leq$2 µg/ml corresponded to S and $>$2 µg/ml to non-susceptible (NS) [10,11]. Strains displaying a MIC variation within one dilution were considered as having a similar susceptibility pattern.

Results

The patients' age ranged from 40–83 years old and 75% (9/12) were male. Data such as department of admission, date of first isolation and strain origin are presented in Table 1 and Fig. 1. All patients were iatrogenically immunosuppressed, and 75% died. All fungaemia cases corresponded to episodes of nosocomial infections. The microsatellite genotyping analysis is detailed in Table 1.

Among the 12 patients, two or more isolates were obtained from blood and from additional body locations. We considered isolates as being highly related when their genotypes differed at only a single locus. In the large majority of patients, blood isolates displayed the same multilocus genotype or a genotype that was highly similar to that of isolates from other body locations. In one single case, patient 10, one isolate from urine exhibited a completely distinct

Table 1 *Candida albicans* isolates from blood cultures and from other body sites obtained from 12 patients and respective multilocus genotyping results.

Patient	Isolate no.	Source	Isolation date(day/month)	Microsatellite genotype				MIC mg/ml				
				CAI	CAIII	CAVI	CEF3	FLC	ITC	VRC	AmB	CAS
1	1.1	Blood	21/4	11-11	5-6	7-11	129-143	>64	>8	>8	1	0.5
	1.2	Bronchial washing fluid	22/4	11-11	5-6	7-11	129-143	>64	>8	>8	0.5	1
2	2.1	Lower respiratory secretions	15/4	11-11	5-6	7-11	129-143	>64	4	>8	0.5	0.5
	2.2	Urine	15/4	11-11	5-6	7-11	129-143	>64	4	>8	0.5	0.5
	2.3	Blood	16/4	11-11	5-6	7-11	129-143	>64	4	>8	0.5	0.5
3	3.1	Central venous catheter	02/4	26-28	6-6	7-7	136-145	0.125	0.015	0.015	0.25	1
	3.2	Blood	22/4	26-28	6-6	7-7	136-145	0.125	0.015	0.015	0.25	1
	3.3	Urine	25/4	26-28	6-6	7-7	136-145	0.125	0.015	0.015	0.25	1
	3.4	Blood	30/4	26-28	6-6	7-7	136-145	0.125	0.015	0.015	0.25	1
4	4.1	Blood	30 /8	26-26	6-6	7-7	136-145	32	>8	>8	0.5	0.25
	4.2	Urine	30 /8	26-26	6-6	7-7	136-145	32	>8	>8	0.5	1
	4.3	Blood	08 /9	26-26	6-6	7-7	136-145	32	>8	>8	0.5	1
5	5.1	Blood	20 /9	26-26	6-6	7-7	136-145	>64	>8	>8	1	1
	5.2	Lower respiratory secretions	20 /9	26-26	6-6	7-7	136-145	>64	>8	>8	1	1
6	6.1	Blood	14/4	25-25	6-7	9-14	131-131	0.25	0.015	0.015	0.25	0.25
	6.2	Blood	26/4	25-25	6-7	9-14	131-131	0.25	0.5	0.03	0.5	0.5
7	7.1	Blood	11/7	21-25	6-7	9-14	131-131	0.5	0.03	0.015	0.25	0.25
	7.2	Blood	19/7	21-25	6-7	9-14	131-131	0.5	0.03	0.015	0.25	0.5
8	8.1	Blood	22/10	21-25	6-7	9-14	131-131	>64	>8	>8	0.25	1
	8.2	Pleural fluid	25/10	21-25	6-7	9-14	131-131	>64	>8	>8	0.5	0.5
9	9.1	Blood	26/10	21-26	7-7	9-14	130-131	16	0.06	0.25	0.5	1
	9.2	Blood	05/11	21-26	7-7	9-14	130-131	16	0.06	0.25	0.5	1
	9.3	Urine	08/11	21-25	6-7	9-14	130-131	16	0.06	0.25	0.5	1
	9.4	Lower respiratory secretions	08/11	21-26	7-7	9-14	130-131	16	0.06	0.25	0.5	1
10	10.1	Blood	02/8	11-17	5-5	7-7	126-135	>64	4	>8	0.5	1
	10.2	Urine	02/8	27-46	6-11	20-20	139-144	>64	4	>8	0.5	1
11	11.1	Blood	21/9	29-29	6-6	7-11	129-143	>64	>8	>8	1	1
	11.2	Urine	21/9	28-29	6-6	7-11	129-143	>64	>8	>8	1	1
12	12.1	Catheter	18/5	18-34	6-7	40-40	137-139	>64	2	>8	0.5	1
	12.2	Blood	31/7	18-34	6-7	42-42	137-139	>64	4	>8	0.5	1
	12.3	Lower respiratory secretions	31/7	34-34	6-7	40-40	137-139	>64	0.5	0.03	0.5	0.5
	12.4	Blood	26/8	18-34	6-7	40-40	137-139	>64	>8	>8	0.25	0.25

Strain replacement is highlighted in dark grey; microevolutionary events are highlighted in light grey.

multilocus genotype from the blood isolate, even though they were collected at the same time. Isolate 9.3 from urine displayed a genotype that differed at two loci compared to the isolates obtained from the respiratory tract or blood isolates. This isolate was moderately related to the other isolates from the same patient but was highly related to isolates from patients 6, 7 and 8. In two patients, 11 and 12, minor genetic differences were detected among blood isolates and those from other body sources. In these patients, the isolates were highly related, suggestive of microevolution.

Consecutive blood isolates from the same patient exhibited the same microsatellite genotype in 5 out of 6 patients. The exception was patient 12 where a highly related isolate was observed between two consecutive blood cultures. This was indicative of strain maintenance regardless of antifungal treatment, rather than re-infection by a distinct strain (Table 1). Isolates from patients 3, 4 and 5 showed identical or highly similar microsatellite profiles (Table 1), an interesting fact since these patients had initially been simultaneously admitted in the same intensive care unit (ICU). Isolates from patients 6, 7 and 8 also exhibited identical or highly similar genotypes (Table 1) and were initially admitted to the same ICU, although not concurrently. All isolates from patients 1 and 2, who where in the same ICU in the previous months, displayed the same genotype (Table 1). Sharing of the same strain among different patients is suggestive of hospital-acquired exogenous nosocomial infection with hospital endemic strains.

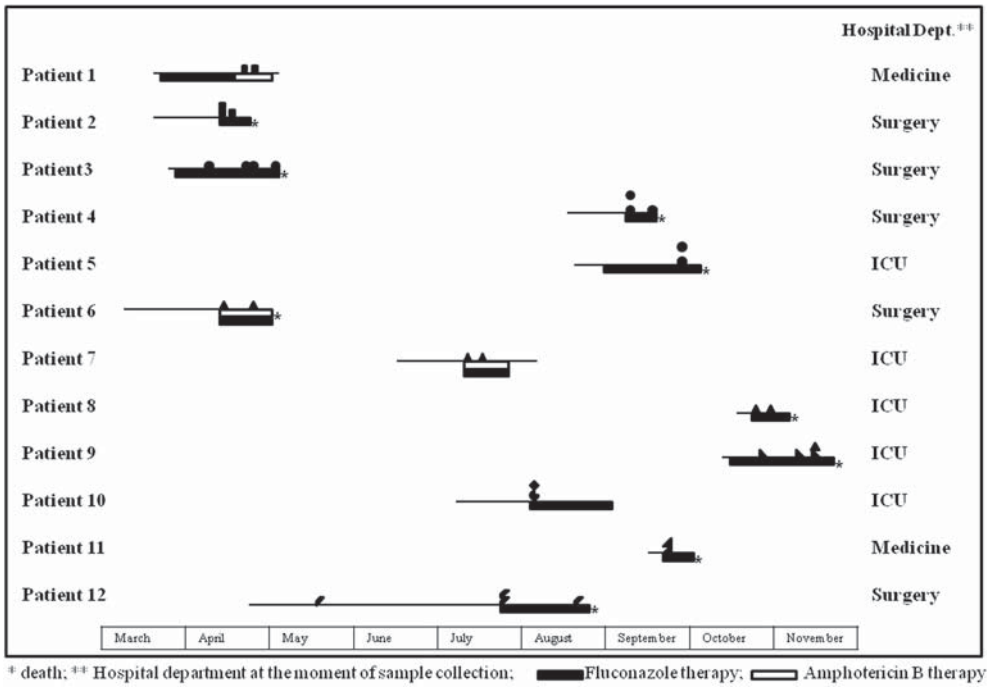

Fig. 1 Schematic representation of patients' clinical data and genetic relatedness of *Candida albicans* strains. The duration of hospitalization (from admission to discharge or death) of each patient is represented by a thick line. Identical symbols represent isolates with the same or highly related genotype.

The antifungal therapy timelines are detailed in Fig. 1. All doses were administered according to the Clinical Practice Guidelines for the management of candidiasis [13]. Four patients (1, 3, 5 and 9) were under antifungal prophylaxis with fluconazole for 2 or 3 weeks prior to the first fungal isolation. Only patient 1, in which fluconazole therapy was replaced by amphotericin B, had a favorable outcome. Two patients (patient 3 and 6), with end-stage neoplasic diseases, were infected with fluconazole-susceptible strains and died due to fungaemia according to their medical records. In patients 3, 4 and 5 who were infected by the same strain, MIC increases were observed. Isolates from patients 4 and 5 that were collected several months after isolates from patient 3 were resistant to azoles (Table 1 and Fig. 1). Similarly, we observed azole cross-resistance in isolates from patient 8 that were collected several months after isolates from patients 6 and 7. All of these isolates had identical genotypes and probably represented the same strain (Table1 and Fig. 1).

All of the isolates analyzed showed low MIC values to amphotericin B and caspofungin ($\leq$1 μg/ml) (Table 1).

Discussion

Nosocomial infections represent an important source of morbidity and mortality in hospital settings [14]. An understanding of pathogen distribution and relatedness is critical for both the epidemiological surveillance of healthcare related infections and for the development of rational pathogen control policies [15]. Pathogen typing allows for the determination of genetically- and epidemiologically-related isolates. The development and implementation of new DNA-based technologies and molecular analyses over the last 3 decades have led to considerable advances in microbial typing approaches [16–19]. The identification of a pathogen's origin, e.g., endogenous *versus* exogenous, and the characterization of consecutive infections by the same organism as relapse or re-infection are critical [19]. Our group has previously developed a microsatellite multiplex PCR strategy with high discriminatory power for typing *Candida albicans* [7]. This methodology was found to efficiently discriminate *C. albicans* strains, as well as *C. glabrata*, *C. parapsilosis* and *C. tropicalis* [17,20,21]. Maintenance and infection by the same strain may indicate that the therapeutic regimen was unsuccessful and that alternative therapies might be required. The results obtained here for successive blood isolates from the same patient suggest the failure of antifungal therapy in these cases. Moreover, fungal strains may invade the host from different body sites, and microevolution, due to strand slippage during DNA replication in the microsatellite region or loss of heterozygosity (LOH), may represent an adaptive fungal response to new environments [7,18]. We detected microevolution events in 3 patients (9, 11 and 12). *C. albicans* colonization and/or infection of different body sites

may represent a predisposing condition or an initial step towards subsequent development of fungaemia [3,22].

All the fungaemia cases considered in this study were of nosocomial origin, according to the accepted definition [8]. Our results clearly demonstrate that isolates displaying the same or highly similar genotypes were obtained from patients who shared the same hospital department of admission which clearly suggests hospital-acquired infections. These observations indicate that the safety measures used between patients most probably failed and that incidents of cross-infection were likely to have occurred.

In our study, fungaemia relapses were frequent, appeared to be caused by the same strain, and were invariably associated to a poor therapeutic outcome. The extension of cross-resistance to azoles detected among fungaemia patients may challenge the large empiric use of fluconazole and how it can lead to the development of dramatic resistance. We observed induction of azoles resistance in two groups of patients (patients 3, 4 and 5 and patients 6, 7 and 8) and the increase in MIC values, over time, in similar strains from different patients suggests that the strains were endemic to the hospital environment for at least several months. This exemplifies the risk of selecting for strains with increased antifungal resistance in the hospital environment.

The molecular identity of fungal isolates represents a key feature for comprehensible therapeutic strategies in a near future; indeed, analysis of dominant genotypes in different geographical regions, distinct clinical samples and separate underlying diseases is now a possibility. Molecular approaches are now available, allowing detailed comparisons between *C. albicans* clinical strains and providing a clear definition of their genetic relatedness.

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Research article

Propofol lipidic infusion promotes resistance to antifungals by reducing drug input into the fungal cell

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Abstract

Background: The administration of non-antifungal drugs during patient hospitalization might be responsible for discrepancies between *in vitro* and *in vivo* susceptibility to antifungals. Propofol is often administered to intensive care units as a sedative.

The purpose of this study was to evaluate the effect of propofol lipidic infusion upon the growth and susceptibility profile of pathogenic fungi.

Candida and *Aspergillus* were studied regarding the ability to grow and its susceptibility profile to antifungals in the presence of propofol infusion (Fresenius®) (1.25, 2.5 and 5 $mg.ml^{-1}$) and its lipidic vehicle. The intensity of fluorescence after staining with FUN1, in the presence and absence of propofol infusion, was determined by flow cytometry. Radioactivity assays were also performed in order to quantify the input of [^{3}H]- itraconazole into the fungal cell in the presence of propofol. Assays were repeated after addition of sodium azide, in order to block efflux pumps.

Results: Propofol infusion promoted budding of *Candida* and the germination of *Aspergillus*, latter forming a lipid layer around the hypha. An increase of minimal fungicidal concentrations regarding both *Candida* and *Aspergillus* strains was found for all antifungals when incubated simultaneously with propofol infusion. A decrease of the intensity of fluorescence of *Candida* cells was systematically observed, as well as a significant reduced intracellular uptake of [^{3}H] itraconazole in cells treated with propofol infusion, even after the blockade of efflux pumps. The results obtained when testing with the lipid vehicle were similar.

Conclusion: Propofol infusion, due to its lipidic vehicle, increased the fungal germination and promoted resistance to antifungals. This effect seems to be related to the reduced access and/or permeabilization to fungal cells by antifungals.

Background

Discrepancies between *in vivo* and *in vitro* susceptibility to antifungals discourage microbiologists and clinicians regarding the routine use of susceptibility testing methods. Although *in vitro* resistance usually correlates with clinical resistance, high susceptibility *in vitro* is not always related to clinical success, particularly for *Aspergillus* spp., most strains being susceptible *in vitro* [1]. Ultimately, the mortality rates are unacceptably high in patients treated with antifungals that showed high *in vitro* efficacy [1,2]. Propofol is an intravenous hypnotic agent very popular for induction and maintenance of general and intravenous anaesthesia. It is commonly administered in Intensive Care Units to critically ill patients, often under mechanical ventilation, which represent a high risk group for health care related infections. The use of propofol has been previously associated to an increased risk for infection, although some controversy still remains [3,4]. It was proposed a low risk of contamination whenever providing standard hygienic precautions [3,5]. Nevertheless, other observations described the lipid emulsion of propofol as a good culture medium to support the growth of *Candida albicans* and *Escherichia coli* [6,7]. Additionally, other reports associated post surgical infections with the extrinsically contamination of propofol infusion [5,8]. Propofol has also been shown to inhibit a variety of functions of neutrophils *in vitro*, although such effect was not so evident *in vivo* [9].

We have studied the effect of the infusion of propofol and its lipidic vehicle upon antifungal susceptibility of *Candida* and *Aspergillus* spp. A promotion of resistance due to a decreased input of the antifungal drugs was found.

Results

In *Candida* strains, budding and germ tube formation were similar in presence of all tested concentrations of propofol infusion. In non-*albicans* strains, the incubation with 5 $\mu g.ml^{-1}$ of propofol infusion resulted in a significant increased of cells with buds when comparing to control (71.5% ± 7.46 *versus* 26.6% ± 4.15, *C. parapsilosis* n = 5 as a representative example) ($p < 0.001$). Conversely, a significant reduction of germ tube formation was observed in *C. albicans* strains comparing with non-treated yeasts (17.3% ± 6.29, *versus* 76.2% ± 8.69, n = 5) ($p < 0.001$). *Aspergillus* conidia germinated in RPMI 1640 culture medium after 4 to 6 hours, the hyphal form being obtained following 10 to 12 hours. Propofol infusion supported the germination of all *Aspergillus* strains, showing similar values to those obtained in plain RPMI 1640 medium. A significantly higher percentage of germination of *A. fumigatus* conidia was found in PBS plus propofol and plain propofol infusion in comparison with plain PBS (21% ± 3.6 *versus* 6% ± 1.6, n = 5) ($p = 0.047$) following 6 hours at 37°C. Longer incubation periods of conidia with propofol infusion showed lipidic drops or a lipidic layer around the hypha, in all assays and with all strains, which remained present following several washings steps (Figure 1).

The result of MIC determination revealed that all fungal strains were susceptible to the tested antifungals. Propofol infusion or its vehicle, at the tested concentrations, consistently promoted an increase of MFC mean values for *Candida* and *Aspergillus* strains(Table 1), this effect being dose-dependent and statistically significant ($p < 0.001$); such effect was invariably observed with all strains of *Candida* and *Aspergillus* and with all antifungals, in some cases the mean values increasing over 4 fold. MFC values in the presence of 5 $mg.ml^{-1}$ of propofol infusion or its vehicle increased at least 2 dilutions in all strains (above the error rate of the method) for fluconazole and voriconazole, more than 3 dilutions for amphotericin B and 4 to 5 dilutions for posaconazole and itraconazole. There was no large variability between the tested strains.

Flow cytometry analysis of *Candida* blastoconidia resulted in 98% of cells stained with FUN1, even after the incubation of sodium azide (Figure 2b). However, after treatment with propofol infusion and stained with FUN1, a non-stained sub-population of cells, similar to autofluorescence, of around 15% was revealed soon after 90 minutes (Figure 2c). This fact was unrelated with the incubation with sodium azide at a concentration able to block the efflux pumps (Figure 2c). The azole resistant strain of *C. albicans* 95–190 (with overexpression of efflux pumps genes), after treatment with propofol infusion and stained with FUN1, revealed a non-stained sub-population, similar to other susceptible strains, even after the blockade of the efflux pumps with sodium azide. This effect was similar in presence of all the tested concentrations of propofol.

After 1 hour, the accumulation of [^{3}H]-labelled itraconazole was detected in blastoconidia cells (control cells) (Figure 3). However, a decrease of 39% in intracellular [^{3}H]-labelled itraconazole was seen when yeast cells were incubated with propofol lipid infusion (Figure 3). This effect was similar in presence of the different tested concentrations of propofol lipidic infusion and did not increase following the pre-incubation with sodium azide (Figure 3).

The results obtained in the different assays were similar when performed with propofol lipidic vehicle.

Discussion

Propofol administered in a lipid-based emulsion to patients has been shown to represent an excellent vehicle for supporting the growth of various microorganisms

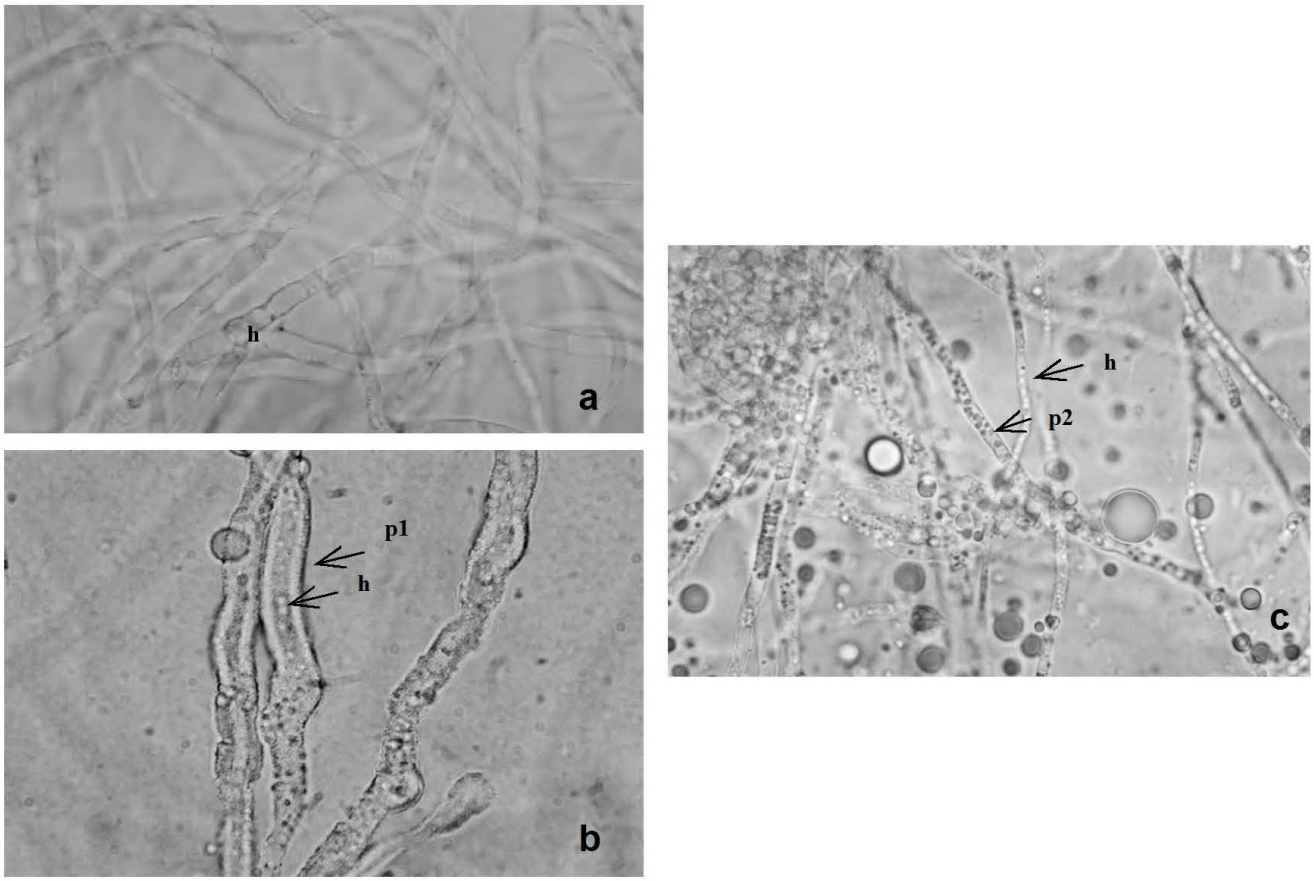

Figure 1
Representative example of *Aspergillus fumigatus*, after 24 hours of incubation: a. non-treated cells; b. cells treated with 5 mg.ml^{-1} of propofol infusion; c. cells treated with 5 mg.ml^{-1} of propofol infusion and washed thrice in sterilized water. (**h** hypha; **p1** lipidic layer around hypha; **p2** lipidic drops).

[6,7]. In the present study we clearly showed that budding of *Candida* spp. was promoted when incubated in the presence of propofol infusion, significantly more than in a culture medium. *Aspergillus fumigatus* germinate faster than the other *Aspergillus* species, possibly being associated to higher pathogenicity [10]. We showed that propofol infusion also supported mould germination and growth, apart from yeasts. It is important to emphasize that the propofol concentrations used in all assays are within the plasma levels often achieved in clinical practice [11,12].

Due to the opacity of propofol infusion or its lipidic vehicle it was not possible to determine MIC values. As the conventional susceptibility phenotypes refer to MIC values, we could not establish the corresponding phenotypes after incubation with propofol. In the case of amphotericin B (a fungicidal drug to both *Candida* and *Aspergillus*) MFC, determined after culture in agar solid medium, usually corresponds to MIC values; for the other antifungals, MFC values are usually located within one or three dilutions above the MIC. Some *C. albicans* and *C. tropicalis* showed trailing phenotype (about 2 dilutions) for azoles. This common effect may influence the evaluation of MFC values. However, trailing is only azole dependent and was not affected by the presence of propofol infusion. Nevertheless, we could observe a significant promotion of the MFC value in presence of propofol infusion, in some cases up to 4 dilutions, suggesting that the strains became quite tolerant to the effect of all antifungals. FUN1 is a fluorescent probe that is converted by metabolically active yeasts in intracytoplasmic vacuolar structures [13]. We have used this probe to study susceptibility of yeasts after incubation during one to two-hours with the antifungals; a higher intensity of fluorescent in susceptible strains, decreasing in resistant strains was described [14,15]. The decrease of the intensity of fluorescence could be explained by the presence of energy-dependent efflux pumps, which were reverted with sodium azide or several modulators [13,14]. In the present study, the viable cells showed a decrease of

Table 1: Minimal fungicidal concentration (MFC) values of *Candida*and *Aspergillus* strains to AMB (amphotericin B), FLC (fluconazole), ITC (itraconazole), VRC (voriconazole) and PSC (posaconazole), determined by CLSI protocols, in the absence and presence of propofol infusion

		Strains (n)						
		MFC values* range μg.ml^{-1}						
Antifungals	Propofol(mg.ml^{-1})	*C. albicans* (5)	*C. tropicalis* (5)	*C. parapsilosis* (5)	*C. glabrata* (5)	*A. fumigatus* (5)	*A. flavus* (4)	*A. niger* (4)
AMB	0	0.25	0.25–1	0.06–0.125	0.25–0.5	0.25–0.5	0.5–1	0.06–0.25
	1.25	0.5–1	1	2	2	1–4	1–>16	0.25–1
	2.5	0.5–2	1–2	2	2	2–4	1–>16	0.25–1
	5	2–4	2	2–8	4–8	4–16	4–>16	0.5–2
FLC	0	8–16	4–8	8–16	16	nd	nd	nd
	1.25	16–>64	>64	>64	>64	nd	nd	nd
	2.5	16–>64	>64	>64	>64	nd	nd	nd
	5	16–>64	>64	>64	>64	nd	nd	nd
ITC	0	nd	nd	nd	nd	0.5–16	0.125–0.25	0.125–16
	1.25	nd	nd	nd	nd	>16	2–>16	>16
	2.5	nd	nd	nd	nd	>16	8–>16	>16
	5	nd	nd	nd	nd	>16	16–>16	>16
VRC	0	0.06–0.25	0.25–0.5	0.5–1	4–8	0.25–4	0.25–2	0.06–8
	1.25	0.25–>2	>2	>2	>8	2–8	0.25–2	1–16
	2.5	0.5–>2	>2	>2	>8	2–8	0.5–4	1–16
	5	1–>2	>2	>2	>8	2–8	0.5–4	1–16
PSC	0	0.06–0.125	0.03–0.125	0.06–0.125	1–2	0.03–0.25	0.03–0.06	0.06–1
	1.25	1–2	>2	2	2	1–4	0.25–2	0.125–8
	2.5	>2	>2	2	>2	1–4	0.5–2	0.25–16
	5	>2	>2	>2	>2	1–4	0.5–4	0.25–>16

nd not done

* The mean value for each strain was considered

the intensity of fluorescence in the presence of propofol which was not however reverted with sodium azide, even considering the resistant tested strain. Furthermore, cells were washed several times before the addition of the fluorescent probe in order to avoid its binding to propofol infusion. These results support that propofol infusion affected FUN1 access and/or permeabilization, reducing the input of the fluorescent probe into the cell. We had also previously shown that [^{3}H]-labelled itraconazole accumulation could be a useful tool to confirm the existence of efflux pumps in resistant *Candida* strains to azoles [16]. The blockade of efflux pumps with sodium azide resulted in the reduction of input of [^{3}H]-labelled itraconazole in *Candida* cells, after incubation with propofol infusion. In fact, propofol infusion was responsible for the difficult access of itraconazole to the fungal cell.

Attending to the facts that i) the increase of MFC values was similar with all antifungal drugs, including the recently available posaconazole; ii) FUN1 could not stain a considerable percentage of the yeast cells in presence of propofol infusion and iii) a lesser amount of [^{3}H]-labelled itraconazole was present within such cells, even after the addition of sodium azide, we are forced to conclude that the promotion of antifungal resistance in presence of propofol lipidic infusion results from a reduced access and/or permeabilization of antifungal agents into the fungal cells. The same results were obtained when only the propofol vehicle was used, thus we had concluded that the major responsible for such effect was the lipidic nature of propofol infusion. The formation of a lipidic layer surrounding the cells supports such assumption, being responsible for the reduced permeabilization. Several studies reported that the lipidic membrane of fungal cells plays an important role in susceptibility to azoles [17,18]. Fluctuations in the lipidic environment affects, not only drug diffusion but also efflux pumps, coded specially by Cdr1 and Pdr5p, leading to multidrug azole

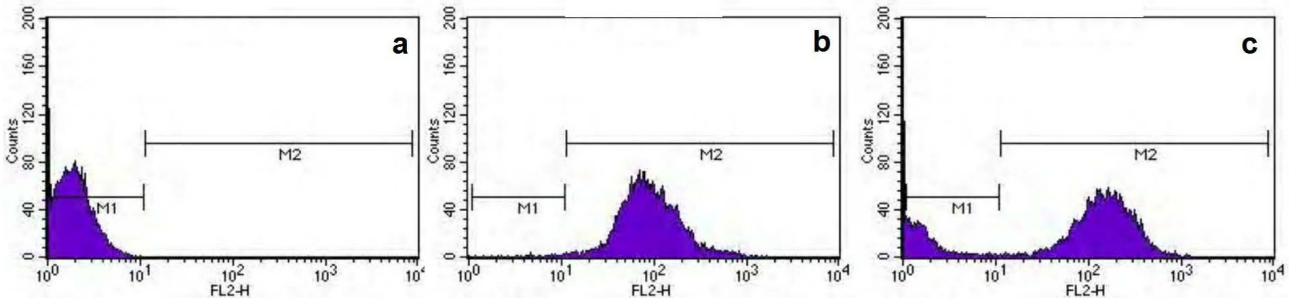

Figure 2

Figure 2
Flow cytometric histograms representing the emitted fluorescence after 90 minutes by: a. non-stained yeast cells (autofluorescence); b. cells treated with sodium azide and stained with FUN1; c. cells treated with sodium azide and 5 mg.ml^{-1} of propofol infusion and stained with FUN1 (strain of *Candida albicans* shown as a representative example).

resistance [19,20]. In our study, as the efflux pumps were blocked with sodium azide, the increased MFC values probably resulted from poor diffusion of the antifungal drugs caused by the lipidic vehicle layer or drops deposited around the cells. The hypothesis that propofol could be binding the antifungal agent in culture medium reducing the free number of molecules available for penetrating the cells was also raised. MFC results were more evident when propofol was associated with itraconazole or posaconazole (increase of 4 to 5 dilutions), both lipophilic drugs. However, we must consider that different concentrations of propofol resulted in similar effects. The hypothesis that the lipidic vehicle of propofol might be playing a role in sterol homeostasis and changing the azole target, should be considered attending to other research [21]. Lipid uptake by the fungal cell was described in the presence of the azoles following longer incubation time (overnight) [21]. Measuring sterols in azole-treated fungi in the presence or not of the lipidic vehicle of propofol could be interesting however, the incubation time with propofol on cytometric and radioactivity studies was very short to allow the incorporation of the lipid in membrane.

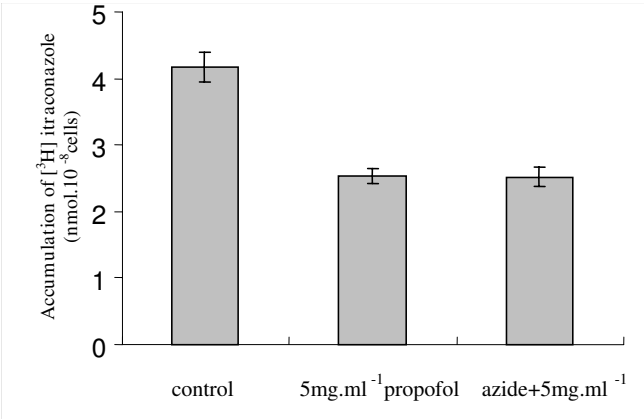

Figure 3
Effect of propofol up on [^{3}H]-labelled itraconazole accumulation in antifungal susceptible strain *C. albicans* ATCC 90028. The accumulation of itraconazole was measured in the absence and presence of 5 mg.ml^{-1} of propofol and after a prior incubation with 0.1 mM sodium azide. Dispersion bars relates to standard deviation.

Conclusion

The assays described in this manuscript provided an opportunity to describe the effect of propofol infusion in antifungal drug resistance. We concluded that propofol infusion, due to its lipidic vehicle reduced the access and/or permeabilization to *Candida* and *Aspergillus* cells to main antifungals administered to patients.

The described effect should raise the alert to a promoted risk of fungal infections in patients receiving propofol infusions, resulting from the fact that fungal strains become increasingly resistant to antifungals.

Methods

Strains

Twenty clinical strains of *Candida* spp. (5 *C. albicans*, 5 *C. tropicalis*, 5 *C. glabrata* and 5 *C. parapsilosis*) and thirteen strains of *Aspergillus* spp.(5 *A. fumigatus*, 4 *A. flavus* and 4 *A. niger)* were studied. *C. albicans* 95–190, resistant to azoles by overexpression of efflux pumps genes (CDR1 and CDR2), was used during cytometric approach (strain

kindly gift by Prof. Theodore White). Until testing, yeasts and moulds were kept frozen in Brain-Heart broth (Difco Laboratories, Detroit, MI, USA) with 5% glycerol. For each experiment, the strains were subcultured twice on Sabouraud agar (Difco) at 35°C, 48 hours for *Candida* and 7 days for *Aspergillus*.

Drugs and Chemicals

Propofol infusion Fresenius® (Kabi, France) at stock concentration of 1% was used. Propofol vehicle (soya bean oil, egg lecithin, glycerol, sodium hydroxide and sterile water) was also assayed. Fluconazole and voriconazole were obtained from Pfizer (Groton, CT, USA), amphotericin B from Bristol-Myers Squibb (New York, USA), itraconazole from Janssen-Cilag (Beerse, Belgium) and posaconazole from Shering-Plough (Kenilworth, NJ, USA). Antifungals drugs were maintained in stock solution at -70°C until use. [^{3}H]-labelled itraconazole was supplied by Janssen-Cilag. Sodium azide was purchased from Sigma (Sigma-Aldrich, Germany).

Growth assays

After cultivation of *Candida* and *Aspergillus* strains in Sabouraud agar medium (Difco, Detroit, MI, USA), a 5 × 10^6.ml^{-1} blastoconidia or conidia suspension of *Candida* and *Aspergillus* was prepared in phosphate buffer saline (PBS) (Sigma) and 100 μl were added in two parallel serial dilutions of propofol infusion (stock solution at 1%) and its vehicle (both at 0, 1.25, 2.5 and 5 mg.ml^{-1} final concentrations) in RPMI 1640 culture medium (Sigma), PBS and plain propofol infusion in a final volume of 500 μl. RPMI is a hydrophilic medium, however, solubility problems were not found. For *Candida* strains, samples were collected after 3 hours incubation at 37°C, the cells were observed under phase contrast microscopy (Leitz Larborlux K) and the percentage of budding and germ tube formation (for *C. albicans*) were determined [22]. For *Aspergillus*, the percentage of conidial germination was determined after incubation for 6, 12 and 24 hours at 37°C [10].

Susceptibility testing

For *Candida* spp., the minimal inhibitory concentration (MIC) to fluconazole, voriconazole, posaconazole and amphotericin B (tested concentration range: 0.125–64 μg.ml^{-1}, 0.03–16 μg.ml^{-1}, 0.03–16 μg.ml^{-1} and 0.03–16 μg.ml^{-1}, respectively) were determined accordingly the CLSI protocols M27-A2 (formerly NCCLS) [23]. Strains were classified as susceptible (S), susceptible-dose dependent (S-DD) and resistant (R) to fluconazole according to breakpoints defined by CLSI [23]. For voriconazole MICs ≤ 1 μg.ml^{-1} were considered S, MIC = 2 μg.ml^{-1} considered S-DD and MIC ≥ 4 μg.ml^{-1} considered R [24]. Although susceptibility breakpoints have not yet been established for amphotericin B and posaconazole, strains with MIC ≤ 1 μg.ml^{-1} were considered susceptible [25,26]. Minimal fungicidal concentration (MFC) to all antifungals was also determined. The content of each well containing drug concentrations to and higher than the MIC, and also the positive growth control were transferred to Sabouraud dextrose agar plates and incubated at 35°C for 48 h, as previously described [27]. The MFC was the lowest drug concentration that killed ≥ 99% of the final inoculum.

For *Aspergillus* spp., the CLSI protocol M 38-A was used to determine MIC values of voriconazole, posaconazole, itraconazole and amphotericin B (all antifungals tested concentration ranged 0.03–16 μg.ml^{-1}) [28]. Although the unavailability of breakpoints for *Aspergillus* species, we followed several researchers that consider values of MIC ≤ 1 μg.ml^{-1} as susceptible [29,30]. MFC values were determined, as previously described [31], and defined as the lowest drug concentration that showed either no growth or fewer than three colonies to obtain approximately 99 to 99.5% killing activity.

The susceptibility tests to the antifungals mentioned above were repeated in the presence of the propofol infusion or its vehicle in three distinct concentrations (1.25, 2.5 and 5 mg.ml^{-1}). Since propofol infusion and its vehicle are opaque solutions, making impossible MIC determination, the content of each well containing antifungal + propofol drugs was cultured for MFC determination and values compared with the MFC to antifungals alone.

Flow cytometry analysis

Yeast cells were incubated at 150 rpm, overnight, until late exponential growth, in Sabouraud broth (Difco) at 37°C. Yeasts cells were harvested after centrifugation and a 1 × 10^6 cells.ml^{-1} suspension was prepared in PBS supplemented with 2% glucose (GH solution) and later divided into aliquots of 1 ml. The cells were then incubated with different concentrations of propofol infusion (0, 1.25, 2.5 and 5 mg.ml^{-1}) at 37°C for 90 minutes and afterwards washed thrice, ressuspended in sterile water supplemented with 2% glucose and stained with 0.5 μM FUN1 (Molecular Probes, Europe BV, Leiden, Holland) for 30 minutes at 37°C. A Beckman Coulter XL-MCL flow cytometer (Beckman-Coulter Corp., Hialeah, FL, USA) equipped with a 15 nm argon laser was used. From each suspension 30000–50000 cells were analysed. The intensity of fluorescence emitted by cells treated with propofol infusion was determined at FL2 (575 nm) and compared with non-treated cells (control). In parallel experiments, yeast cells were treated with 0.1 mM sodium azide during 30 minutes, prior to incubation with propofol, as previously described [16], in order to block efflux pumps; thereafter, the same flow cytometry analytical protocol was used.

Intracellular accumulation of [^{3}H]-labelled itraconazole

Candida cells were initially incubated under similar conditions as previously described for flow cytometry assays. The cells were harvested by centrifugation at 5000 rpm for 10 minutes at 4°C, washed thrice, ressuspended in PBS at a final concentration of 2.5×10^8 cells.ml^{-1} and incubated with 0, 1.25 and 5 mg.ml^{-1} of propofol infusion at 37°C, with continuous shaking at 300 rpm for 30 minutes [16]. Parallel experiments were prepared, but also involving a pre-incubation of the yeasts cells with sodium azide at 0.1 mM. The cells were washed thrice and [^{3}H]-labelled itraconazole was added to yeast suspensions at a final concentration of 3 μM, as previously described[16]; the cells were incubated in glass vials at 37°C, with continuous shaking (300 rpm), during 1 hour and then harvested by centrifugation at 5000 rpm for 10 min at 4°C, washed thrice with 3 ml of ice-cold PBS containing 10 μM unlabelled itraconazole. The pellets were later ressuspended in 500 μl of PBS and the radioactivity was determined, following the addition of a scintillation cocktail (Optiphase "Hiphase3", Perkin-Elmer), in a liquid scintillation counter (LKB Wallac, 1209 RackBeta).

Lipidic vehicle experiments

All the described experiments were repeated in the presence of the propofol lipidic vehicle used in Fresenius® formulation (soya bean oil, egg lecithin, glycerol, sodium hydroxide and sterile water).

Statistical analysis

The effects of different concentrations of propofol upon germination of fungal cells and MFC values of the distinct antifungals were compared using one-way analysis of variance (ANOVA) and Student's *t*-test. Significance was accepted at $p < 0.05$. The SPSS 14.0 program for Windows was used to perform the statistical analysis. All susceptibility experiments were run in duplicate and growth and radioactivity assays in triplicate.

Authors' contributions

SCO and RA designed the study, performed the cytometric and radioactivity assays and wrote the manuscript. ASD performed growth and susceptibility assays. CPV and AGR also helped in the design of the study and draft the manuscript.

All authors read and approved the final manuscript.

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Candida krusei reservoir in a neutropaenia unit: molecular evidence of a foe?

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Abstract

Candida krusei has been documented as an emerging pathogen causing nosocomial outbreaks. The consecutive isolation of *C. krusei* strains in three patients admitted to the same hospital department within 2 months lead us to consider the possibility of an outbreak. Additionally, *C. krusei* isolates were collected from the room surfaces, whereas another isolate had been recovered from the blood of one patient 2 years before. *Hin*fI DNA restriction endonuclease-based analysis of all *C. krusei* isolates was performed and restriction profiles were compared. Surprisingly, isolates from different patients were unrelated, whereas isolates from biological products of the same patient showed indistinguishable *Hin*fI restriction patterns and were similar to those obtained from the surrounding environment of the respective patients. The study approach revealed the endogenous origin of the *C. krusei* infectious episodes observed and demonstrated that, subsequent to colonizing a patient, *C. krusei* can be involved in infectious episodes distant in time. The hypothesis of an outbreak was excluded, although we believe that the methodology employed in the present study represents a valuable tool for diagnostic and epidemiological surveys.

Keywords: *Candida krusei,* haematological patients, health care related infections, molecular typing, mt DNA Restriction, Endonuclease Analysis, nosocomial outbreak

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Introduction

In the last two decades, invasive fungal infections in hospitalized patients have increased significantly worldwide. According to data obtained from the USA and Europe, *Candida* species represent, respectively, the fourth and sixth most frequent cause of invasive healthcare-related infections [1,2], accounting for 8–15% of all episodes of sepsis acquired in hospital settings [3]. Inherent to these types of infection are the extremely high morbidity and mortality rates, particularly among immunocompromised patients [4,5].

Fluconazole is one of the antifungal agents mostly used in both prophylactic and therapeutic protocols. Fluconazole prophylaxis has been associated with a decrease in the prevalence of *Candida* species such as *Candida tropicalis* and *Candida albicans*, and to an increase in that of *Candida krusei* and *Candida glabrata* [3]. *C. krusei* presents intrinsic resistance to fluconazole and, to some extent, reduced susceptibility to amphotericin B [6].

Infectious outbreaks in hospitals, especially in intensive care units, represent a serious health problem and are mainly due to *Candida lusitaniae* [7], *C. albicans* [8], *Candida parapsilosis* [9], and *C. krusei* [10,11]. Many factors may account for their occurrence (e.g. barrier loss, lack of proper infection control measures by health care workers when managing patients, resistance to prescribed antifungal drugs, as well as insufficient drug levels).

Molecular methods represent a powerful tool to clarify transmission pathways in health care facilities (i.e. to investigate the occurrence of possible outbreaks). Techniques such as karyotyping, restriction fragment length polymorphism analysis by pulsed-field gel electrophoresis, southern blot

hybridization, PCR fingerprinting and randomly amplified polymorphic DNA fingerprinting have been extensively used for *Candida* typing [12–16]. Restriction endonuclease analysis (REA) has been described in the last decade as a valuable tool for *Candida* spp. characterization. REA of the mitochondrial DNA (mtDNA) was first applied in the biotechnology industry in order to characterize yeast strains used for wine fermentation [17,18] and, more recently, to discriminate between *Candida* clinical strains [19–22]. The data obtained demonstrate the relevance of using molecular genetic methods in many different areas, including taxonomic, ecological and clinical surveys.

Recently, we were challenged by a hypothetical outbreak as a result of *C. krusei* in the neutropaenia unit of the Haematology Department of Hospital S. João, Porto, Portugal. Within a short period of time, several *C. krusei* isolates were cultured from biological products of three patients. In addition, *C. krusei* was found in the surrounding environment of the patients. All isolates were compared using mtDNA REA, which is a convenient and powerful tool that allows valid comparisons between isolates of the same yeast species.

Patients and Methods

Patients

Patient A was a 41-year-old male with acute lymphoblastic leukaemia (T/NK) diagnosed in July 2006. In August 2008, the patient was initially treated with amoxicillin and ciprofloxacin because of undetermined fever. When symptoms remained unchanged, a myelogram was performed; a first relapse of the haematological disease was diagnosed. He was admitted to the neutropaenia unit for salvage intensive chemotherapy with fludarabine, idarubicin and ara-C, followed by growth factor (G-CSF), administered through a central venous catheter. Upon the onset of the aplastic period (28 August), he received antimicrobial treatment for 2 weeks [ciprofloxacin, amoxicillin-clavulanic acid, imipenem, acyclovir and fluconazole (200 mg/day)]. At the time of fungaemia detection, he displayed the following haematologic parameters: white blood cells 0.03×10^9 cells/L, haemoglobin level 8.5 g/dL and blood platelets 11×10^9 cells/L. As soon as *C. krusei* was identified, the patient was started on caspofungin for 12 days without improvement; the treatment was then changed to amphotericin B.

This patient had previously developed a fungaemia episode as a result of *C. krusei* during chemotherapy in 2006 and a corresponding isolate had been stored at −70°C.

The patient remained in room number 1 from August to October 2008.

Patient B was a 53-year-old male with non-Hodgkin's lymphoma, who was admitted to room number 2 for 3 weeks in September 2008. He was administered cyclophosphamide, doxorubicin, vincristine, and prednisone, followed by G-CSF. On 12 September 2008, he was started on caspofungin but, as a result of sustained fever on day 5, treatment was changed to voriconazole, completing 14 days of antifungal therapy. He never received fluconazole. At that fungaemia episode, he displayed the following haematologic parameters: white blood cells 2.41×10^9 cells/L, haemoglobin 9.3 g/dL and blood platelets 73×10^9 cells/L.

Patient C was a 60-year-old male with acute myeloid leukaemia secondary to myelodisplastic syndrome; he was admitted to room number 2 for 3 weeks in October 2008, subsequent to patient B. He received Ara-C, etoposide and doxorubicin for 5 days, followed by another 5 days of Ara-C; he was also prescribed amoxicillin, ciprofloxacin and allopurinol. In addition, prophylactic treatment included imipenem, vancomycin, fluconazole and acyclovir from 29 September to 20 October 2008. On that latest date, he displayed the following haematologic parameters: white blood cells 0.6×10^9 cells/L, haemoglobin 9.8 g/dL and blood platelets 24×10^9 cells/L.

Patient D served as a typing control; one single *C. krusei* isolate was recovered from the patient's bronchial secretions in September 2008 upon his admission to the Internal Medicine Department.

Clinical strains

Eighteen *C. krusei* isolates were collected from different clinical specimens (blood, urine, stools and bronchial secretions) of the above mentioned patients admitted to the neutropaenia unit within a 2-month period. Additionally, a previous *C. krusei* isolate (2006) from a blood culture of patient A was included in the study, as well as a *C. krusei* isolate obtained from the control patient D. All isolates were identified using the automatic system Vitek2 YBC identification cards (BioMérieux, Paris, France), stored at −70°C in Brain Heart infusion (Merck KGaA, Darmstadt, Germany) with 10% glycerol and sub-cultured twice on Sabouraud agar (Merck KGaA) to ensure purity prior to experimental assays.

Environmental strains

Several environmental samples were collected from the patients' rooms (1 and 2) using Sabouraud agar contact plates (Merck KGaA); two *C. krusei* isolates were cultured: one from the bedside table of patient A (room 1) and another from the bed of patient C (room 2). The isolates were characterized and stored as described above for clinical

samples. Air samples were collected by filtration with a MAS-100 Eco instrument (Merck Eurolab, Dietlikon, Switzerland), containing Sabouraud agar plates (Merck KGaA); no *C. krusei* isolates were obtained.

Antifungal susceptibility testing

Voriconazole (Pfizer, New York, NY, USA), posaconazole (Schering-Plough; Kenilworth, NJ, USA), amphotericin B (Bristol Meyers Squibb, New York, NY, USA), caspofungin (Merck, Rahway, NJ, USA) and anidulafungin (Pfizer) stock solutions were prepared according to CLSI protocols (M27-A3) [23] and maintained at −70°C until use. Minimal inhibitory concentration (MIC) of each antifungal drug was determined according to CLSI protocol M27-A3 [23].

Total genomic DNA extraction

Yeast cells were cultured overnight at 30°C in 10 mL of YPD liquid medium, with continuous orbital shaking at 180 r.p.m., and subsequently collection by centrifugation. Total DNA was extracted using phenol:chloroform:isoamyl alcohol 25:24:1, precipitated using 100% ice-cold ethanol and redissolved in 200 μL of TE buffer. The DNA was treated with 20 μg of RNase (Applichem, Darmstadt, Germany), incubated at 37°C for 30 min to 1 h. For final precipitation, 20 μL of 4 M ammonium acetate, pH 4.8 (Sigma-Aldrich, Munich, Germany) and 600 μL of ice-cold 100% ethanol (Applichem) were added and samples were incubated overnight at −20°C. The DNA was re-dissolved in TE buffer 1x, assessed in a biophotometer 6131 (Eppendorf, Hamburg, Germany) and adjusted to a final concentration of 2.0–2.5 μg/μL. To assay DNA integrity, approximately 3–5 μg of DNA was run in agarose gel (1%, w/v) (Sigma-Aldrich) in TBE buffer 1x and stained with ethidium bromide (0.5 mg/mL) (Applichem). DNA samples were stored at −20°C for subsequent use.

REA of mt DNA

For each sample, a reaction mixture was prepared containing 1x *Hin*fI enzyme reaction buffer (Metabion, Planegg, Germany), 1 μg/μL RNase, 0.5 U/μL *Hin*fI restriction enzyme (Metabion), approximately 25–30 μg of total DNA and DNAse-RNAse free water up to 20 μL final volume; reaction tubes were incubated overnight at 37°C. Restriction was ended upon *Hin*fI inactivation by incubating for 20 min at 80°C. The total reaction mixture was run on a 1% agarose gel (20 cm × 24 cm) at 120 mV for 3–5 h, stained with ethidium bromide (0.5 mg/mL) and the DNA visualized under UV light.

Restriction patterns were analyzed using the UVIDOC 12.6 software for Windows (Topac Inc., Cohasset, MA, USA) and the resulting groups of strains were compared.

Results and Discussion

There have been an increasing numbers of reports describing non-*albicans Candida* hospital outbreaks. Given that *C. krusei* is not the main pathogen causing nosocomial infections, the detection of simultaneous episodes at the neutropaenia unit of our hospital lead us to consider the possibility of an outbreak.

The *C. krusei* isolates were all susceptible to all the antifungals assayed (fluconazole was not tested because *C. krusei* presents intrinsic resistance to this agent). Variations in the antifungal MICs for different isolates were not significant. The MICs of amphotericin B were from 0.06 to 1 mg/L; for caspofungin from 0.125 to 1 mg/L; for anidulafungin 0.06 mg/L; for voriconazole from 0.25 to 2 mg/L; and for posaconazole from 0.03 to 0.5 mg/L.

The routine biochemical identification protocols or antifungal susceptibility profiles are usually not sufficient to either corroborate or exclude an outbreak hypothesis. REA for *Candida* species was first described by Scherer and Stevens [24] who considered this method to be an extremely valuable tool for epidemiological studies. Fujita *et al.*[21] described *Hin*fI restriction patterns as exhibiting a superior discriminatory power among distinct *Candida* isolates compared to patterns obtained with other enzymes such as *Eco*RI or *Msp*I. Additionally, Sancak *et al.*[22] established a correspondence of almost 100% between the results obtained with *Hin*fI restriction endonuclease-based analysis and PCR methodologies. In the present study, a total of 22 *C. krusei* isolates (20 clinical, two environmental) were analyzed using REA. A high number of *C. krusei* isolates was obtained from different biological products of patient A in distinct periods of time. All of them showed the same restriction pattern, including the isolate recovered in 2006 from a blood culture (A_{BC1}) (Fig. 1, lanes A_{BC1}, A_{BC2} and A_{CVCII}), indicating that they are the same strain. Most certainly, this patient harbors a reservoir of *C. krusei* and was colonized throughout a long period of time, as described similarly for *Acinetobacter* [25] and *Pseudomonas* [26]. This is the first report, to our knowledge, describing a long-lasting colonization by *C. krusei.* These results have implications in terms of prophylactic measures (i.e. fluconazole is not recommended in a patient with previous isolation of *C. krusei*). Other antifungals, such voriconazole or amphotericin B, are more likely to be efficient in these cases [27,28].

The *C. krusei* isolates from each patient yielded distinct *Hin*fI restriction patterns, suggesting that the isolates were different strains (Fig. 1, lanes A_{BC2}, B_{BS}, C_S), at the same time discarding the hypothesis of an outbreak in the neutropaenia unit where the patients were admitted. The *C. krusei* strain isolated from

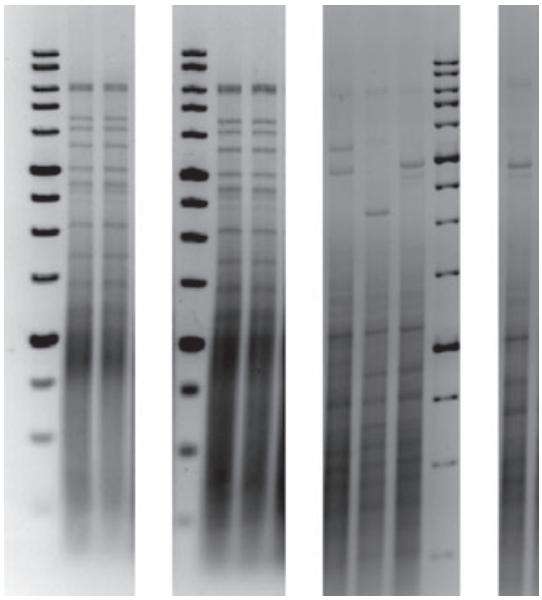

FIG. 1. Restriction endonuclease patterns of *Hin*fI-digested DNA obtained after agarose gel electrophoresis. Each pattern corresponds to *Candida krusei* isolates from patients A, B, C and D (upper case letters), from different biological products (BC, blood cultures; CVC, central venous catheter; BS, bronchial secretions; S, stools), and to *C. krusei* isolates from the room environment of patients A and C (A_E and C_E, respectively); M, molecular weight marker (1 kb DNA ladder; Metabion).

patient D showed a pattern distinct from those isolated from the remaining patients (Fig. 1, lane D_{BS}), as expected.

The two environmental *C. krusei* isolates collected from the surfaces of the rooms where patient A (A_{E9}) (room 1) and patients B and C (C_E) (room 2) had stayed were different, as depicted in Fig. 1. This excludes the possibility of different *C. krusei* strains being transmitted as a result of patient handling by health care workers. However, both the environmental and the clinical *C. krusei* isolates associated with the same patient displayed an undistinguishable restriction pattern, as shown in Fig. 1 (lanes A_{CVC11} and A_{E9} vs. C_S and C_E), suggesting a putative environmental reservoir and the possibility of subsequent transmission from it to other patients. Our results emphasize the need to enhance preventive infection control measures, both when handling the patients directly and when cleaning patients' facilities, particularly those admitting neutropenic patients. Indeed, a study by Berrouane *et al.* [29] described the relatedness among *C. krusei* clinical isolates obtained from different biological products from both patients and health care workers.

As described in the present study, we were able to exclude the hypothesis of an outbreak occurring in the neutropaenia unit throughout the time period considered, supporting the usefulness and suitability of the REA methodology. Moreover, the present study provided very useful information concerning *C. krusei* reservoirs existing in patients and their surrounding environment.

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Transparency Declaration

The authors declare that there is no source of funding and no potential conflicts of interest.

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Virulence Attenuation of *Candida albicans* Genetic Variants Isolated from a Patient with a Recurrent Bloodstream Infection

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Abstract

The incidence of *Candida albicans* infections and the relapse episodes after antifungal treatment have increased in recent decades. Recurrences are mainly due to the persistence of the original infecting strain that may present genetic and genomic rearrangements during interaction with the host, reflecting strain adaptation. In this study, four isolates recovered from a patient during recurrent candidemia episodes were genotyped by microsatellite length polymorphism (MLP) and by multilocus sequence typing (MLST) and found to be genetic variants of the same strain. Using experimental mouse infections, a progressive reduction in the virulence of the four isolates was observed, with the first two isolates more virulent than the third and fourth. Additionally, in the mouse model, the first isolate resisted host control more efficiently, resulting in higher kidney fungal burdens and necrosis as compared to the third isolate. The resolution of inflammation was delayed in mice challenged with the first isolate and the message for IFN-γ and TNF-α in the spleen was lower within the first few hours post-infection. Original and recurrent isolates also displayed different phenotypes regarding activity of secreted enzymes and response to stress agents. Overall, the comparative analysis indicated that the virulence decrease of these isolates was related to a lower ability to resist to the host anticandida effector mechanisms. We showed for the first time that *C. albicans* genetic variants of the same strain, sequentially isolated from an immunocompromised patient, underwent adaptations in the human host that resulted in virulence attenuation when tested in mice.

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Introduction

Candida albicans is a common colonizer of the human gastrointestinal, respiratory, and reproductive tracts. However, in immunocompromised patients, this species is one of the most important opportunistic fungal pathogens, being responsible for both superficial and systemic infections [1,2]. Despite the prevalence of *Candida* in the hospital environment and the poor outcome of this infection, the pathways involved in clearance of mucocutaneous and systemic infections have not been fully defined and the majority of the clinical studies focus on epidemiology, diagnosis and therapeutic management [3].

Molecular epidemiology studies showed that *C. albicans* isolates exhibit a high level of genetic diversity. Microsatellite length polymorphism (MLP) and multilocus sequence typing (MLST) have been used to discriminate *C. albicans* strains and to detect small genetic changes or microvariations that may be indicative of adaptability processes [4–7]. Typing of multiple *C. albicans* isolates from the same patient obtained in longitudinal studies, or in surveillance cultures from different anatomical sites, showed a tendency towards the maintenance of the same strain during the infection process [6,7]. This view of a monoclonal infecting population has recently been extended by the demonstration of colony-to-colony variation in *C. albicans* primary isolations in samples from patients with vaginal and oral infections [8]. Nevertheless, the referred study also showed that strain variability in primary cultures from established infections is much lower than from healthy individuals, suggesting that the infecting population results from the selective proliferation of one or more clones that were present in the mixed commensal population before the establishment of the infectious process. Observations on the genetic and phenotypic variation in *C. albicans* populations showed higher rates of chromosome-level genetic variations during passage in the mouse relatively to in vitro growth [9], and in strains isolated from the digestive tract of healthy individuals [10]. These genomic alterations may be involved in the generation of new variants within the population that contribute to the adaptation during infection.

Host defense against systemic candidiasis relies mainly on the ingestion and elimination of *C. albicans* by cells of the innate immune system, in particular macrophages, monocytes, and neutrophils [11–15]. Activation of leukocytes by *C. albicans*, triggers the release

of pro-inflammatory cytokines (Th1 and Th17 responses), such as IFN-γ, TNF-α, IL-1β, IL-6, and IL-17 that in turn activate phagocyte effector functions to eliminate the invading yeast [12,16–19]. In contrast, anti-inflammatory cytokines (Th2 response) such as IL-4 and IL-10 have immunosuppressive effects. Thus, the balance between pro- and anti-inflammatory cytokines is decisive in determining whether the host defense system is overwhelmed or able to eliminate the fungal pathogens [14,20–25]. Although the status of the host immune system is the major factor balancing the transition from commensalism to pathogenicity [26], *C. albicans* expresses several virulence attributes that contribute for its successful behavior, both as a commensal colonizer and as a pathogen [27]. One of its major virulence traits is the ability to reversibly switch from unicellular budding cells to filamentous forms and the yeast uses this attribute during an infection, not only to invade tissues, but also to escape intracellular phagocyte death by inducing hyphal growth inside the phagosome, resulting in the destruction of the macrophage [28–30].

In the present work, we assessed the virulence of *C. albicans* isolates from a patient with recurrent candidemia treated during a period of four months with fluconazole. Typing of the isolates determined that they were variants of the same strain and it was observed that those genetic variants were progressively less virulent to mice. With this study, we show for the first time that variants of the same strain, recovered from a patient during recurrent infections, differ considerably in terms of their capacity to produce disease when tested in an immunocompetent host.

Results

Candida albicans isolates from a case of recurrent candidemia are genetic variants of the same strain

C. albicans isolates used in this study were recovered from cases of recurrent candidemia (Table 1). The four isolates analysed in more detail were obtained from patient 1 and collected within a period of four months. Isolate 124A was the first recovered and, despite the patient's treatment with fluconazole, three other isolates, 140, 140A, and 144, were sequentially collected. All four isolates were found to be resistant to fluconazole, presenting MIC values >64 μg/ml. These isolates showed the same multilocus genotype by MLP, except 140A, which presented a loss of heterozygosity (LOH) at CAI microsatellite (Table 1). MLST analysis also showed that the isolates were closely related, although presenting minor differences, resulting in different diploid sequence types (DSTs). To gain a better insight into the genetic proximity of these four isolates, a similarity UPGMA dendrogram based on MLST data was constructed. Strains isolated from other patients were also typed and included in the Clustal analysis to generate a more robust tree. This analysis showed that all isolates from patient 1 grouped closely, within a p distance value lower than 0.02, and with a nodal support value of 1 after 1000 bootstrap replications (Fig. 1), indicating that the four isolates could be considered undistinguishable, or genetic variants of the same strain.

Mouse virulence of the isolates decreased progressively

In view of the genetic similarity of the isolates, the question of whether they also behaved identically regarding virulence towards a healthy host was raised, and the mouse model of i.v. disseminated candidiasis was used to assess virulence.

Survival analysis of mice inoculated with 2×10^6 yeast cells showed that the first isolates (124A and 140) were the most virulent, while the last ones (140A and 144) were less virulent (Fig. 2A). Comparing mice infected with the first isolate (124A) with mice infected with the third (140A), or with the fourth (144), the overall differences in survival were highly significant ($P=0.0034$ and $P=0.0002$). In fact, mice infected with 124A, 140, 140A or 144 presented median survival times of 4.0, 4.5, 8.0, and 13.5 days, respectively. Differences in virulence between isolates 124A and 140A were confirmed using a lower inoculum (Fig. 2B). When testing reference strain SC5314 with the same inoculum, all mice succumbed during the first two days post-infection, in accordance to what is described in the literature [31].

Table 1. Microsatellite genotypes and diploid sequence types (DSTs) obtained by MLP and MLST analysis of the clinical isolates used in this study.

Patient	*Isolate*	*Isolation Data*	*Local of isolation*	*MLP Genotypes*			*MLST DST*	*Clade*
				CAI $(CAA/G)_n$	**CAVI $(TAAA)_n$**	**CEF3 $(TTTC)_n(TTC)_n$**		
1	DBC-124A	18-05-04	Catheter	18–34	12–12	137–139	1282	16
	DBC-140	31-06-04	Blood	18–34	12–12	137–139	1283	16
	DBC-140A	31-07-04	Bronchial secretions	34–34	12–12	137–139	1284	16
	DBC-144	26-08-04	Blood	18–34	12–12	137–139	1285	16
2	DBC-154	20-09-04	Blood	26–26	7–7	135–146	1277	4
4	DBC-141	02-08-04	Blood	12–17	7–7	126–135	1278	8
5	DBC-165A	22-10-04	Pleural fluid	21–25	9–15	131–131	1279	11
6	DBC-155	21-09-04	Blood	29–29	7–11	129–143	1280	15
7	DBC-164	22-10-04	Blood	26–28	7–7	136–145	1281	4
8	DBC-7J	-	Vaginal	30–30	19–23	126–126	1286	5
	DBC-8J	-	Vaginal	30–32	19–23	126–126	1287	1
	DBC-9J	-	Vaginal	30–32	19–23	126–126	1288	1
9	DBC-22J	-	Vaginal	23–27	18–21	135–136	1289	S*
	DBC-23J	-	Vaginal	23–27	21–21	135–136	1290	S*

The corresponding date and local of isolation, as well as clade assignment based on MSLT, are also shown.
- data unknown; DST – diploid sequence type; S* - singleton.
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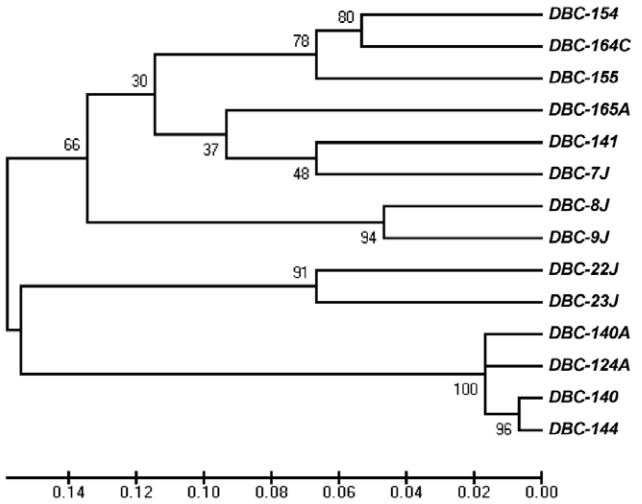

Figure 1. *C. albicans* strain clustering. Similarities between MLST data were analyzed in terms of p distance with MEGA version 4.0 and nodal support values, after 1000 bootstrap replications, were calculated and are depicted on the UPGMA dendrogram.
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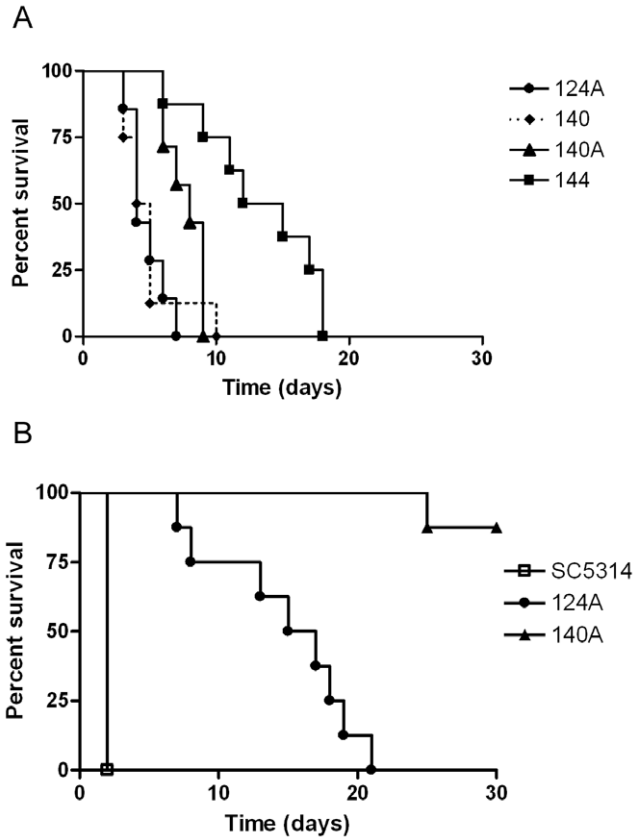

Figure 2. Survival of BALB/c mice following i.v. infection with *C. albicans* strain variants. Mice were infected i.v with (A) 2×10^6 cells of isolates 124A, 140, 140A or 144 or (B) 1×10^6 cells of SC5314, 124A or 140A and the condition of the mice were assessed daily for 30 days.
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These results demonstrated that genetic variants of the same strain, recovered from the same patient during recurrent infections, progressively reduced their virulence when tested in an immunocompetent host.

Decreased survival of inoculated mice is correlated with high kidney fungal burden and necrosis

In order to understand the mechanisms underlying the differences observed in mouse survival, the most virulent isolate (124A) and the isolate with the most significant decrease in virulence (140A) were further studied. A comparative analysis of organ fungal burdens, cytokine expression and histopathology of mice inoculated with these isolates was performed up to the seventh day post-infection. Kidney fungal burden increased from days one to three, decreasing significantly on the seventh day, for mice infected with 140A (Fig 3). At day three post-infection, kidney colony counts from mice infected with 124A were around 10-fold higher when compared to mice infected with 140A, and by day seven this difference was even higher, to nearly 22 fold. On the contrary, splenic and hepatic colony counts declined progressively in all mice to nearly undetectable levels, showing no significant differences between the two isolates (results not shown). Differences regarding organ distribution are in accordance with the known predilection of *C. albicans* for kidney colonization, after mouse systemic infection [32].

The higher mouse susceptibility to infection with isolate 124A was also evident in H&E and PAS stained histologic sections of the kidney (Fig. 4). At day one, and particularly at day three, kidney sections of mice infected with 124A exhibited extensive tissue necrosis and lack of an apparent cellular infiltration (Fig. 4A and 4C). Additionally, in the same period, PAS staining showed a dramatic increase in fungal cell numbers in mice infected with 124A (Fig. 4C). In contrast, kidney sections of mice infected with 140A showed degraded yeast cells, and a marked inflammatory leukocyte influx, indicating a resolving lesion (Fig. 4B and D). At day seven post-infection, in mice infected with isolate 124A, the fungal cells were predominantly in the hyphal form and were apparently intact, forming a clear barrier to the progression of inflammatory leukocytes. On the contrary, kidney histology of mice infected with 140A showed an intermixing of fungal cells with inflammatory leukocytes and degraded fungal cells, suggesting that yeast cell proliferation was controlled (Fig. 4E and F). These results are in accordance with kidney CFU counts obtained previously.

To get a better insight into the nature of the immune response of mice infected with these isolates, spleen expression of IFN-γ, TNF and IL-4 was determined by real-time RT-PCR at one, three, and seven days post-infection (Fig. 5). Cytokine expression showed that at day three, mice infected with the 124A isolate presented significantly lower levels of IFN-γ in comparison with mice infected with 140A. However, by day seven post-infection this difference inverted, and mice infected with 124A presented significantly higher levels of IFN-γ and TNF (Fig. 5A and 5B). For expression of IL-4, no differences were found between the isolates, except at day seven post-infection, when isolate 140A resulted in the expression of slightly higher values (Fig. 5C).

Resolution of inflammation is delayed in mice infected with the primary isolate

A comparative analysis of leukocyte recruitment to the peritoneal cavity of mice infected with 124A or 140A was next performed. Figure 6 shows that *C. albicans* infection stimulated an acute leukocytosis, predominantly due to the recruitment of

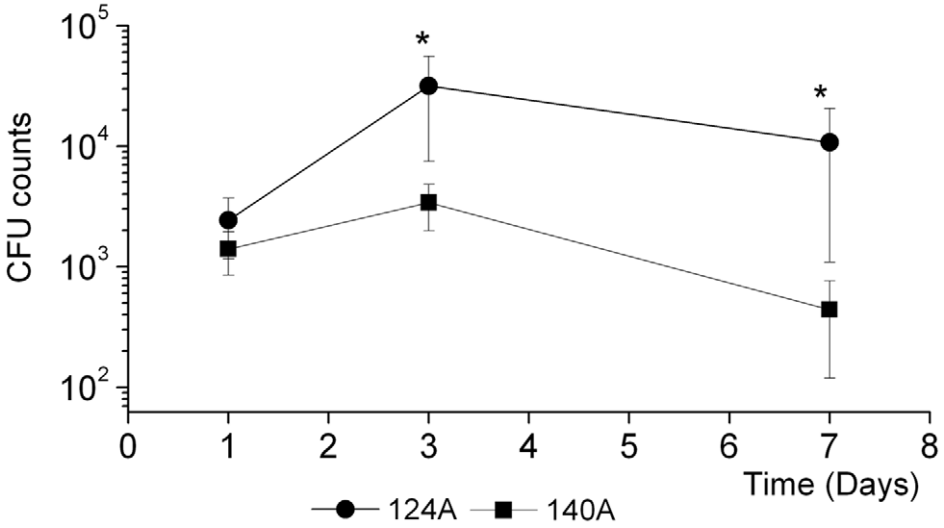

Figure 3. Kidney fungal burden. Groups of four mice infected i.v. with 10^6 *C. albicans* cells were killed at 1, 3 and 7 days after challenge. Organs were homogenized in HBSS and the suspension diluted and cultured on Sabouraud dextrose agar. Results are presented as log of colony-forming units (CFUs). Statistically significant differences between results at each hour of infection as evaluated by Student's *t* test are labeled with single asterisk ($P<0.05$).
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neutrophils, as previously described [33–35]. Counts of the peritoneal exudate leukocytes showed that the number of neutrophils increased more than 190 fold ($P<0.001$) in the infection with isolate 124A, and 158 fold ($P<0.001$) following infection with 140A. Using the set of resolution indices from Bannenberg *et al.* [36], in the time interval between three h (T_{max}) and 20 h (T_{50}), exudate PMNs decreased in number from 13.34×10^6 (Ψ_{max}) to 6.7×10^6 (R_{50}), resulting in a resolution interval (R_i) of 17 h (i.e., 3–20 h), in the infection with isolate 124A. For mice infected with 140A, the Ψ_{max} was much lower, 11.38×10^6, and T_{max} higher (eight hours), resulting in a resolution index of 13 h (i.e., 8–21 h). The macrophage cell population showed a similar kinetics in mice infected with isolates 124A and 140A (Fig. 6B). These results indicated that the two *C. albicans* isolates raise similar patterns of leukocyte recruitment. However, the resolution of inflammation is four hours delayed in mice infected with the primary isolate, 124A.

Subsequent isolates induce reduced macrophage death

Isolates 124A and 140A were tested *in vitro* with a macrophage cell line. The percentage of phagocytosis was approximately 11% for isolate 124A and 17% for 140A, but this difference was not statistically significant ($P=0.076$). Phagocyte death, assessed by the number of PI-positive phagocytes, showed that isolate 124A induced death of about 50% of the macrophages after 4 h of co-incubation (Fig. 7). On the contrary, 140A did not induce a significant change in the percentage of macrophage death during the same period. Differences in macrophage death induced by both isolates were statistically significant after 2 h ($P=0.011$) of co-incubation, and continued after 3 h ($P=0.037$) and 4 h ($P=0.001$).

Subsequent isolates have different phenotypic characteristics regarding activity of secreted enzymes and response to stress agents

Phenotypic characteristics known to contribute to *C. albicans* pathogenicity, such as growth rate, response to stress and activity of extracellular enzymes, were evaluated in the two clinical isolates. No significant differences were observed regarding the ability of the isolates to secrete aspartic proteases (Saps) or in their growth rates at 26, 30 or 37°C in SD and YPD media (results not shown). The extracellular in vitro phospholipase activity, determined as the Pz value, showed that isolate 124A presented a higher activity than isolate 140A (Pz value of 0.52 ± 0.001 for 124A and of 0.86 ± 0.042 for 140A, $P<0.05$).

The behaviour of both clinical isolates showed no significant differences regarding growth in the presence of $CaCl_2$, Caffeine, $MnSO_4$, SDS and ethanol, at all tested concentrations, as well as on SD plates at pH 3.7, pH 5.5 or pH 8.0. Both isolates seemed to be equally resistant to osmotic stress induced by NaCl (1M) and sorbitol (1.2 M). However, in the presence of 20 mM acetic acid, isolate 124A was more tolerant than isolate 140A (Fig. 8). The response to 1.25 mM H_2O_2 oxidative stress of the two isolates showed that 140A was significantly more sensitive to H_2O_2 induced death than isolate 124A, presenting a decrease in viability of around 50% [124A 98% ($\pm$23.3), 140A 52% ($\pm$2.3), $P<0.05$].

Phenotypic characterisation showed that the genetic variants behaved similarly although isolate 124A presented a higher phospholipase activity and was more tolerant to acetic acid and H_2O_2 than 140A.

Discussion

Infections due to *C. albicans* may result from the selective proliferation of a single strain variant present in the commensal population before invasive infection [7,8]. In patients with recurrent infections, three basic scenarios were described: (i) maintenance of that same strain, (ii) maintenance of that same strain undergoing microevolution or microvariation, or (iii) strain replacement [4,6,7,8]. Microvariations are relatively frequent and may occur in response to changes within the host, reflecting strain adaptation. Therefore, with the characterization of strains sequentially isolated from patients with recurrent infections, it is important to evaluate whether these adaptations have consequences in host-pathogen interaction. These aspects are particularly relevant when dealing with commensal organisms. It has long been known that different *C. albicans* strains can exhibit varying

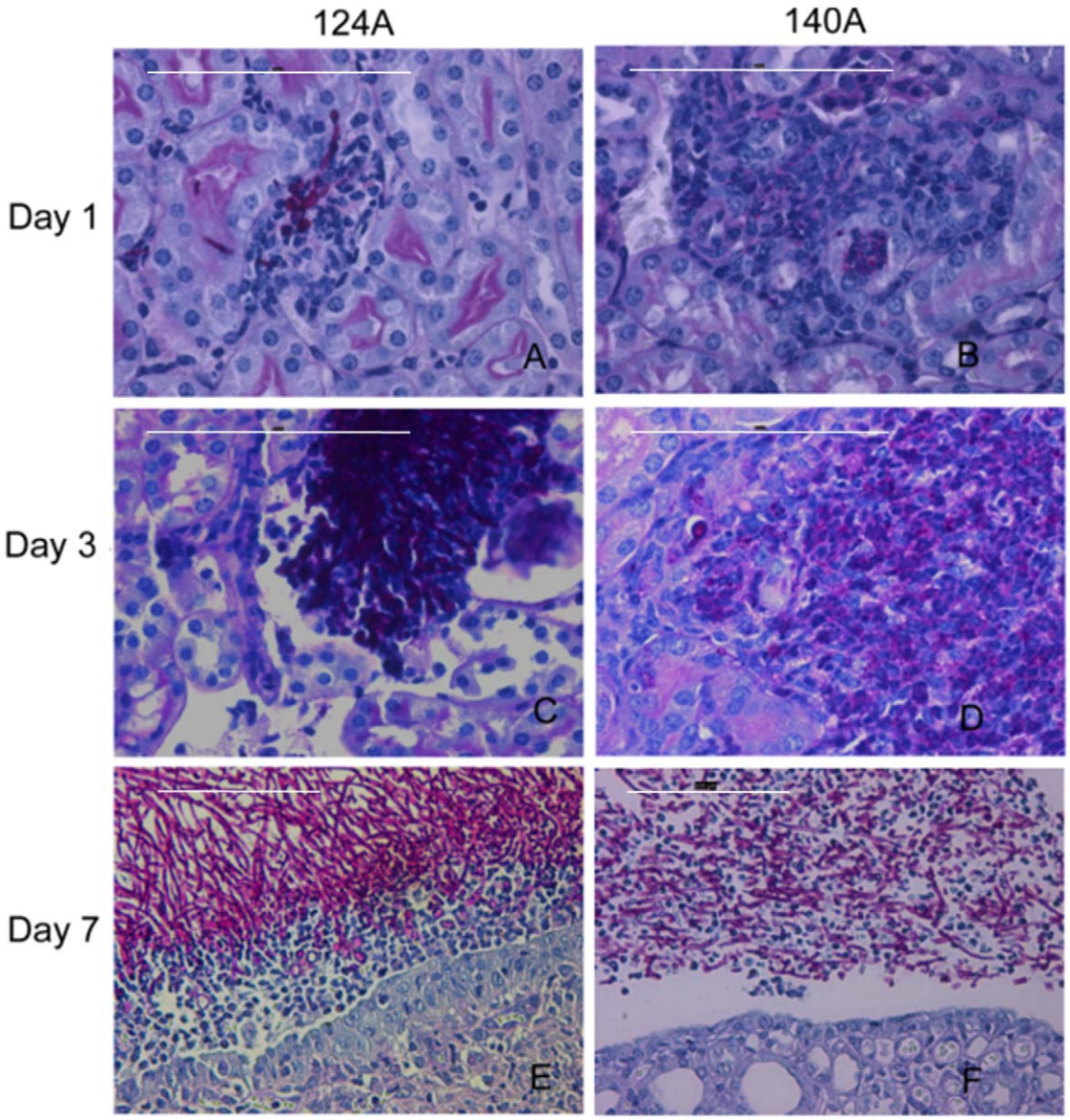

Figure 4. Kidney histology. Representative micrographs of H&E/PAS-stained paraffin sections of kidneys recovered from mice infected with 10^6 yeast cells at days 1 (A and B), 3 (C and D) and 7 (E and F) days post-infection with isolates 124A and 140A.The bar −100 μM.
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levels of virulence when tested both *in vivo* and *in vitro* models [37–41]. However, to the best of our knowledge, no work has characterized the virulence of isolates, and their genetic variants, sequentially recovered from the same patient, as described in the present study.

In this work, four sequential isolates from a patient were genotyped by MLST and MLP and found to be closely related. Cluster analysis, including other strains isolated in the same period from other patients of the hospital, confirmed that the four isolates were very close since they were the only ones to group within a *p* distance value lower than 0.02. According to Odds *et al.* [7] strains that group within this *p* distance value could be considered undistinguishable or variants of the same strain. Interestingly, these isolates showed a clear progressive decrease in virulence in an i.v. mouse model of systemic infection. Since they were variants of the same strain, we concluded that the differences in virulence were not due to different genetic backgrounds of the isolates but to strain adaptation to host changes during the recurrent infections. One might doubt that these changes occurred in such a short time, however recent studies showed that *C. albicans* isolates undergo chromosomal and genetic alterations during a single passage in the mouse [9]. Following these results, the original isolate, 124A, and the first recurrent isolate to present a significant decrease in virulence (140A) were selected in order to understand the mechanisms underlying the observed differences in virulence.

Systemic infection by *C. albicans* is associated with the release of proinflamatory cytokines, including TNF and IFN-γ [42,43]. In this study cytokine quantification showed that although at day seven post-infection the levels of IFN-γ and TNF increased in mice infected with 124A, on day three the levels of IFN-γ were lower, comparing with mice infected with 140A. Moreover, cells from isolate 124A developed long filaments inside the kidney, while cells from 140A appeared as fragmented hyphae intermixed with the inflammatory cells. These observations are in agreement with

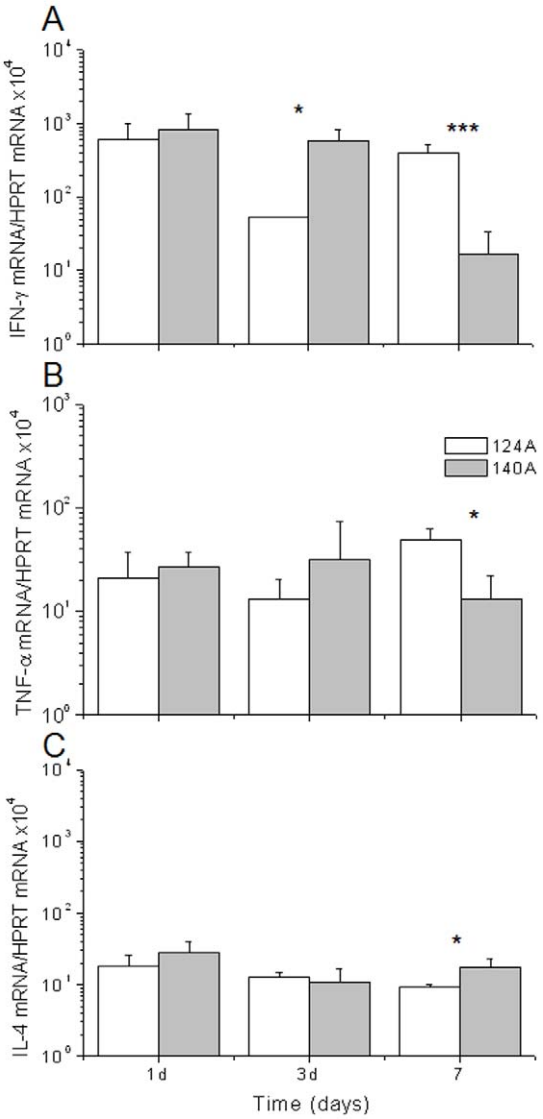

Figure 5. Real time PCR cytokine quantification. RNA was extracted from spleen homogenates in HBSS of mice infected with 10^6 yeast cells of 124A (□) or 140A (■) by using the Trizol method and mRNA levels of IFN-γ (A), TNF-α (B) and IL-4 (C) quantified and expressed as copies per HPRT gene. Statistical significance was calculated by using Student's *t* test and significant differences are labeled with a single asterisk ($P<0.05$) or triple asterisks ($P<0,0001$).
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previous reports, indicating that pro-inflammatory cytokines are important for antifungal effector functions, particularly during the early phase of the inflammatory response [12,33,34,44,45].

The differences observed in the murine virulence study could be due to a differential recognition of the isolates by the host cells, resulting in an impaired inflammatory cellular response, or to an intrinsic higher resistance of isolate 124A to phagocyte killing.

The comparative analysis of leukocyte recruitment to the peritoneal mouse cavity of mice infected with 124A or 140A showed that even though resolution of inflammation was delayed in mice infected with 124A, immunocompetent mice recognized both isolates similarly, invoking an acute neutrophilia, as previously described [35,46,47]. Thus, we tested the hypothesis that the differences in virulence observed in infected mice could be mainly due to an intrinsically higher resistance to phagocyte killing of isolate 124A. One mechanism proposed for the opportunistic *C. albicans* to resist phagocyte killing is by rapidly changing to a filamentous form, allowing the fungal cells to resist ingestion or, if internalized, kill the phagocyte to escape to the extracellular environment [29,30]. This higher resistance was confirmed *in vitro* upon co-incubation with J774 macrophages cell line. Both isolates were equally recognized and phagocytosed, but 124A cells induced a much higher macrophage death than 140A cells. The observation that 124A resisted more efficiently to the presence of H_2O_2, a compound present in the hostile environment of the phagolysosome, and presented a higher activity of secreted phospholipases, also favored its resistance to phagocyte induced death.

Overall, this comparative analysis demonstrated that the virulence decrease of isolate 140A was related to its lower ability to resist to anticandida effector mechanisms, what explains the lower kidney CFU's, the absence of long filaments in kidney histology and in vitro assays, and the faster spontaneous resolution of acute inflammation. We believe that the four isolates from this patient are genetic variants of a strain that, upon interaction with the host, adapted to differences in the microenvironment. It is likely that, as the patient became immunocompromised, the host environment became less stressful, and adaptation resulted in a progressive decrease in virulence that was evidenced when tested in an immunocompetent host. Several works analyzing rates of genetic and genomic alterations and their possible consequences to microbe fitness propose that for opportunistic pathogens, such as *C. albicans*, these alterations favor the commensal state rather than the infectious [9,48,49]. Additionally, Cheng *et al.* [50] isolated a *C. albicans* variant with attenuated virulence after passages through mice, which could also be considered in agreement with the commensal theory proposed.

This study is the first to show a decrease in virulence of genetic variants of the same strain sequentially isolated from a human patient, suggesting that *C. albicans* is able to adjust to the host, favoring commensalism rather than increase of virulence. The ability of *C. albicans* to adapt to and change its virulence in immunocompromised hosts can be a strategy of this organism to maintain its host alive and prolong its own survival.

Materials and Methods

Yeast strains and typing

C. albicans clinical strains (14 isolates) used in this study were collected from nine patients with recurrent infections attending the same hospital (Table 1). The four isolates analysed in more detail were from a patient with gastro-intestinal cancer who had been under chemotherapy (patient 1). This patient was submitted to surgical intervention and presented two sequential bloodstream infections in a period of four months (Table 1). All the isolates and the reference strain SC5314, were maintained on Sabouraud agar plates at 4°C and cryopreserved in 30% glycerol (wt/wt) at −80°C.

Strain typing was performed by using microsatellite length polymorphism (MLP) and multilocus sequence typing (MLST), the more discriminatory typing methods for C. albicans. For MLP analysis polymerase chain reaction (PCR) amplification with CAI, CAVI, and CEF3 markers was performed as described by Sampaio *et al.* [6] and by Bretagne *et al.* [51]. PCR products were run in an ABI 310 Genetic Analyser (AB Applied Biosystems) and fragment sizes were determined automatically using the GeneScan 3.7 analysis software. MLST typing was based on sequence analysis of DNA fragments from the six housekeeping *C. albicans* genes, *ACC1*, *ADP1*, *GLN4*, *RPN2*, *SYA1*, and *VPS13*, as previously reported [52]. The

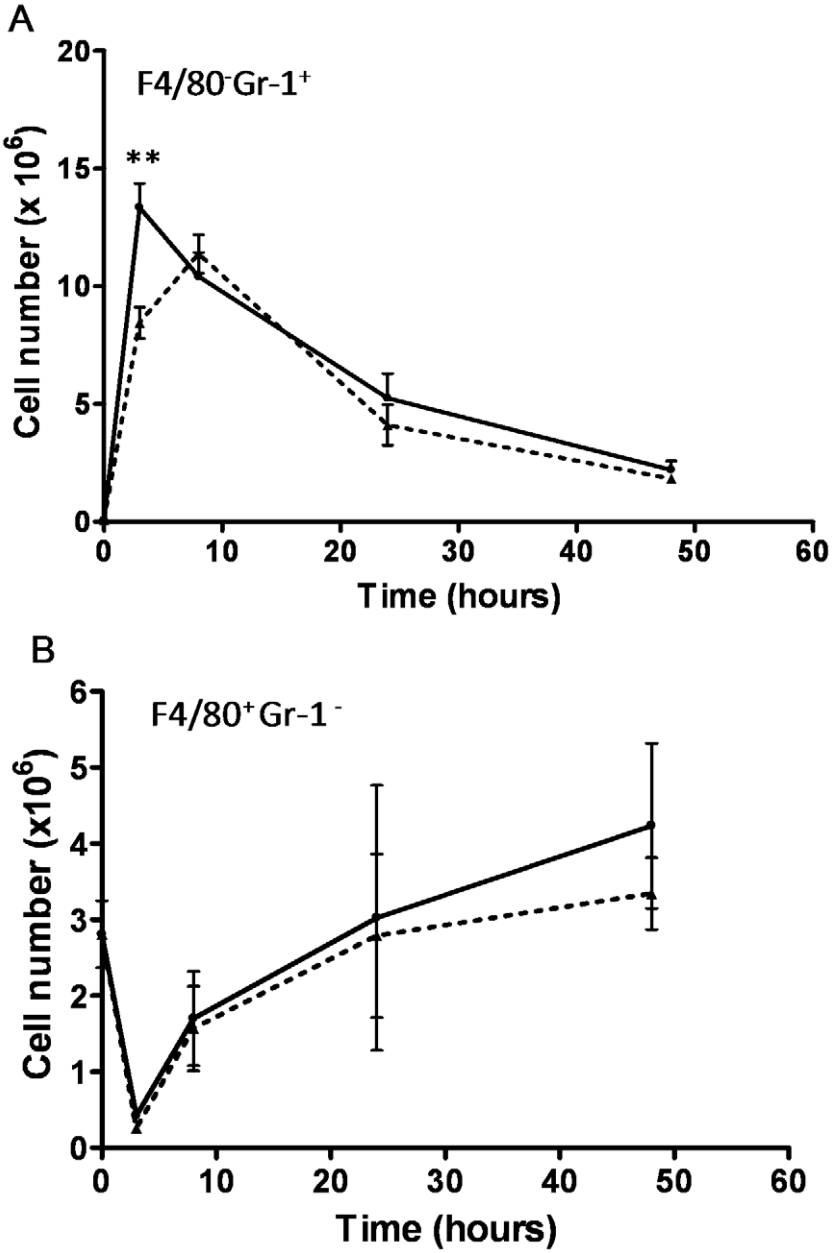

Figure 6. Intraperitoneal inflammatory response to *C. albicans* strain variants. Kinetics of neutrophils (A) and macrophages (B) in the peritoneal cavity following i.p. infection of BALB/c mice with 10^7 cells from strains 124A (solid line) and 140A (dashed lines). Cells were recovered by peritoneal lavage, and counting of leukocytes was performed by flow citometry. Statistically significant differences between results at 3, 8, 24 and 48 hours of infection, as evaluated by Student's *t* test, are labeled with double asterisks ($P<0.001$).
doi:10.1371/journal.pone.0010155.g006

diploid sequence types (DST) obtained were deposited in the *C. albicans* MLST database (http://calbicans.mlst.net/).

Similarities between MLST sequence data were analyzed in terms of *p* distance with MEGA version 4.0 [53], as described by Odds *et al.* [7]. Nodal support, after 1000 bootstrap replications, was also calculated and depicted in the UPGMA dendrogram.

Mice and *C. albicans* hematogenously disseminated infection. Female BALB/c mice 6 to 8 weeks old were obtained from Charles River (Barcelona, Spain) and kept under specific pathogen-free conditions at the Animal Facility of Life and Health Sciences Research Institute (Braga, Portugal). The present study was conducted under the guidelines and approval of the Research Ethics Committee of the same Institute.

To evaluate the virulence of the isolates mice were injected intravenously (i.v.) in the lateral tail vein with 2×10^6 cells of each of the four isolates studied in more detail, in 0.5 ml PBS. For preparation of inocula, cells unfrozen from the original stock were grown in Winge medium (0.2% glucose and 0.3% of yeast extract) at 26°C, to maintain the conidial morphology [54]. In each experiment, all isolates were tested simultaneously and inocula were confirmed by CFU counting of the suspensions used to infect mice. Animal welfare was assessed twice daily during 30 days.

For assessment of organ fungal-burdens and cytokine quantification mice were separated in groups, four mice in each cage, and i.v. infected with a lower inoculum, 10^6 yeast cells. At days 1, 3, and 7 post-infection, mice from a cage were sacrificed and their

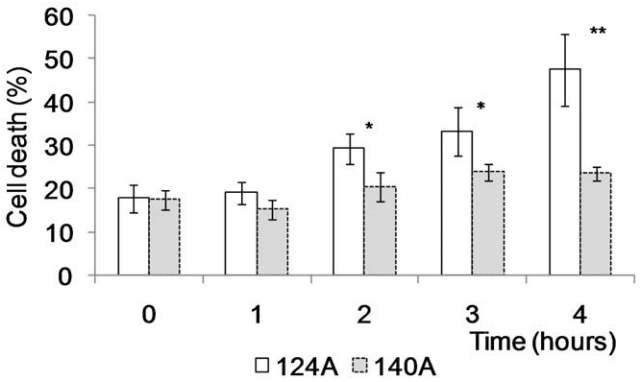

Figure 7. *In vitro C. albicans* macrophage killing. Cells from the macrophage cell line J774 were incubated with *C. albicans* isolates 124A or 140A cells in a ratio of 1:5 (E:T) and dead macrophages identified after incubation with 1 μg/ml of propidium iodide under the florescence microscope. Statistically significant differences between results at each hour of co-infection, as evaluated by Student's *t* test, are labeled with single asterisk ($P<0.05$).
doi:10.1371/journal.pone.0010155.g007

kidneys, livers, and spleens aseptically processed. Organs were homogenized in 2 ml of Hanks Balanced Salt Solution (HBSS) from Invitrogen, diluted, and cultured on Sabouraud agar at 37°C. The results of organ fungal burden were expressed as log CFU/ml of homogenate. Prior to processing, spleens were divided in half to allow simultaneous analysis of fungal colony counts and cytokine quantification.

Cytokine quantification

RNA was isolated from the spleen homogenate obtained previously. Briefly, 200 μl of the organ homogenate were centrifuged at 6000 rpm at 4°C and the pellet resuspended in 0.5 ml of Trizol reagent. After 5 minutes of incubation at room temperature 0.1 ml of chloroform was added, tubes were agitated and incubated on ice for 15 minutes. Samples were then centrifuged at 12000 g for 15 minutes at 4°C and the aqueous phase recovered. RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol, and samples centrifuged at 12000 g for 10 minutes at 4°C. RNA pellet was washed once with 0.8 ml of 70% ethanol and air-dried. RNA was resuspended in 10 μl of ultra-pure water, quantified in the NanoDrop 1000 R Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, NC), and stored at −80°C at a concentration of 200 ng/μl.

Total RNA was reverse transcribed in a thermocycler My Cycler Thermal Cycler (Bio-Rad, Hercules, CA) by using the Superscript Kit II and Oligo dT (Invitrogen). The cDNA was subjected to real-time RT-PCR reactions for quantification of mRNA levels of TNF, IFN-γ, IL-4, and the housekeeping gene mHPRT by using the LightCycler (Roche, Basel, Switzerland), and the LightCycler FastStart DNA Master Hybridization Probes kit. Probes and primer sequences used to amplify the cDNA, as well as the specific annealing temperatures are described in Botelho *et al.* [55].

Histology

Kidneys excised from infected mice were fixed in 10% phosphate-buffered formalin, embedded in paraffin, sectioned, and stained with periodic acid-Schiff (PAS) stain after hematoxylin-Eosin (H&E) staining, according to Kretschmar *et al.* [56].

Quantification of *in vivo* acute inflammatory response

To quantify the cellular acute inflammatory response to isolates 124A or 140A, mice were intraperitoneally (i.p.) injected with 10^7 *C. albicans* cells and killed after 3, 8, 24 and 48 h [56]. The inflammatory infiltrate was collected by lavage with ice-cold PBS [57]. Quantification of leukocyte sub-populations in the peritoneal lavage fluids was performed by flow cytometric analysis (FACScan) based on the expression of F4/80, a marker associated with the macrophage lineage [58], and GR1, a marker associated primarily with the granulocyte lineage [59]. The following monoclonal antibodies (mAbs) were used in the cytometric analysis (Becton-Dickinson, San Jose, CA) using CELLQUEST software (Becton-Dickinson): Phycoerythrin (PE) conjugated anti-mouse F4/80 antigen (clone BM8), and FITC anti-mouse Ly-6G and Ly-6C (Gr-1) (RB6-8C5) (BD Pharmingen). To characterize the resolution of inflammation the following quantitative indices were used: (i) the magnitude of PMN tissue infiltration (maximal PMN, Ψ_{max}); (ii) the time interval when numbers of PMN reach Ψ_{max} within exudates (T_{max}); (iii) the time point (T_{50}) when PMN numbers reduce to 50% of Ψ_{max} (R_{50}); and (iv) the resolution interval (R_i), the time interval from the maximum PMN point (Ψ_{max}) to the 50% reduction point (R_{50}) [i.e.T_{50}-T_{max}] [36].

Macrophage culture and phagocytosis assays

The mouse macrophage-like cell line J774 (ATCC TIB-67) was cultured at 37°C in 5% CO_2 in Dulbecco's Modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal calf serum (FCS) (Valbiotech), 2 mM L-glutamine, 1 mM sodium pyruvate, and 10 mM HEPES. Macrophages were plated at a concentration of 5×10^5 cells/ml into 24-well tissue culture plates (Orange) containing a 13 mm diameter coverslip (Nunc) in each well and incubated overnight in 5% CO_2 at 37°C. *C. albicans* isolates were grown overnight at 26°C in Winge medium,

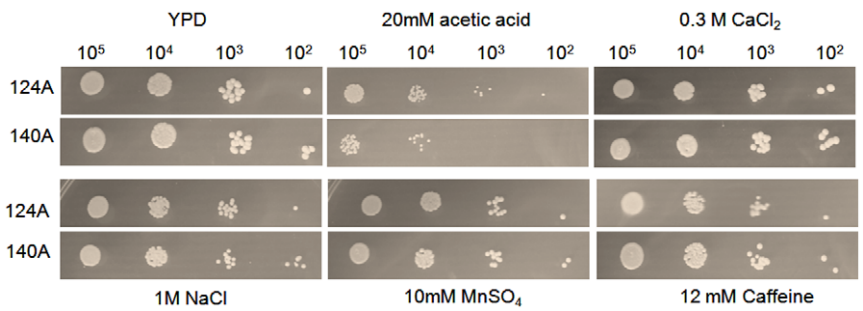

Figure 8. *In vitro* susceptibility assay. Growth of 124A and 140A yeast cells at 37°C for 48 h on YPD and YPD containing 20mM acetic acid, 0.3M $CaCl_2$, 1M NaCl, 10mM $MnSO_4$, and 12mM caffeine. Drop tests were performed by spotting 10 μl of 10^5, to 10^2 cells/ml dilutions.
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recovered by centrifugation at 5000 rpm and washed twice in sterile phosphate buffered saline (PBS).

Phagocytosis was assessed at a 5:1 *C. albicans*/macrophage ratio, and the number of internalized *C. albicans* cells determined in a phase-contrast microscope (Leica DMRB) after 30 minutes of co-incubation [60,61]. Percentage of phagocytosis was determined as the number of internalized cells/number macrophages ×100. At least 300 cells were counted.

Macrophage death assessment was determined by incubating macrophages and yeast cells, as previously described, and cells stained with 1 μg/ml propidium iodide (PI) after 1, 2, 3, and 4 h of incubation. Images were taken in ten independent fields using a Leica DM5000B fluorescence microscope. Percentage of dead phagocytes was determined as the number of PI positive macrophages/number macrophages counted ×100 [62]. At least 300 cells were counted.

Phenotypic screening and susceptibility assays

For the determination of growth rates, a pre-culture was prepared incubating *C. albicans* isolates over night at 30°C in liquid SD (0.17% of YNB, 0.5% of $(NH_4)_2SO_4$, 2% of glucose) and YPD (1% yeast extract, 2% peptone, 2% glucose) media and then a dilution was prepared in fresh medium to start with a OD_{600} of 0.05. Growth rates were determined at 26°C, 30°C and 37°C by measuring OD_{600} every hour until the culture reached stationary phase.

Extracellular proteolytic activity (Saps) of the isolates was assessed in BSA solid and liquid medium according to Monod *el al.* [63]. *C. albicans* isolates were also screened for production of extracellular phospholipase activity by growing them on egg yolk agar and measuring the size of the zone of precipitation by the method of Samaranayake *et al.* [64]. Phospholipase activity (Pz value) was calculated as the ratio of the diameter of the colony and the diameter of the colony plus that of the precipitation zone. Since Saps and phospholipases are inducible enzymes these tests were performed with freshly unfrozen cells from the original stocks.

Strain sensitivity to osmotic, acidic and oxidative stress was determined by incubating the yeast cells on SD agar plates containing several stress conditions: 50 and 100 μg/ml SDS; 1 M NaCl, 7% and 11% Ethanol; 1.2 M Sorbitol; 20 and 50 mM Acetic Acid; 12 mM Caffeine; 0.3 M $CaCl_2$; 10 mM $MnSO_4$, 0.2 M LiCl; and also SD at pHs 3.75, 5.5 and 8. Cells grown overnight in YPD medium were recovered by centrifugation at 5000 rpm and washed in PBS. Drop tests were performed by spotting 10 μl of 10^5 to 10^2 cells/ml dilutions onto the prepared plates that were incubated at 26, 30 and 37°C, for 3 days. Susceptibility to H_2O_2 was assessed by incubating the yeast cells in a solution of 1.25 mM H_2O_2, for 60 minutes at 30°C, and viability measured by CFU counts on YPD D.

Statistical analysis

Unless otherwise stated, results shown are from one experiment, representative of three independent experiments. Statistical significance of results was determined by unpaired Student t-test and survival data were analyzed with the log-rank test, using the GraphPad Prism 4 Software (GraphPad Software, Inc., La Jolla, CA, USA). Results were considered statistically significant with *P* values of less than 0.05.

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Author Contributions

Conceived and designed the experiments: PS AGC JP CP. Performed the experiments: PS MS AC FEA SCdO. Analyzed the data: PS AC JCG CP. Contributed reagents/materials/analysis tools: AGC JP CP. Wrote the paper: PS AGC JP CP.

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Disclaimer: The views expressed in this letter are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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Colonization of central venous catheters in intensive care patients: A 1-year survey in a Portuguese university hospital

To the Editor:

Patients admitted at intensive care units (ICUs) often require central venous catheterization. Catheter colonization may lead to bacteremia and, eventually, end up as a catheter-related bloodstream infection.[1] To review the etiology and antimicrobial susceptibility pattern of central venous catheter (CVC) isolates from ICU patients, a retrospective study of the clinical database of Hospital of S. João, in Porto, was performed during a 12-month period. All positive results from the culture of distal tips of CVCs removed from ICU patients and antimicrobial susceptibility pattern of isolates were reviewed.

From 1482 cultures of CVC distal tips performed during the study period, 647 positive results (43.7%) were found. As expected,[2] coagulase-negative staphylococci (54.7%), mainly *Staphylococcus epidermidis* (40.2%), were the most common isolates; *S aureus* (12.8%), Enterobacteriaceae (8.6%), *Candida* spp (7.0%), *Pseudomonas aeruginosa* (6.2%), enterococci (4.8%) and *Acinetobacter baumannii* (2.8%) followed in the ranking. Of major concern was the resistance rate of *S aureus* to methicillin (80%), of *Enterococcus faecium* to vancomycin (85.7%), of *P aeruginosa* to meropenem (34.8%) and imipenem (65.2%) and of *A baumannii* to both carbapenems (94.4%).[3-7] Interestingly, no resistance to azole antifungals (fluconazole, itraconazole, voriconazole, posaconazole) was detected among the low number of tested *Candida* isolates (n = 35), in accordance with previous findings.[8] This fact might result from the limited exposure to antifungal agents in this hospital because no antifungal prophylactic treatment is routinely prescribed to such patients.

Globally, the etiology of CVC colonization in ICU patients was similar to international available data. However, higher levels of antimicrobial resistance were found for *S aureus*, *E faecium*, *P aeruginosa* and *A baumannii* isolates. Routine surveillance for resistance patterns and the prospective evaluation of new therapeutic protocols might be highly advisable in such setting.

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Erratum

At the request of Neghat Lakdawala, the journal wishes to revise our February article, ''Decontamination of laundry at low temperature with CuWB50, a novel copper-based biocidal compound,'' (Am J Infect Control 2009;37:478-83) by removing Ms. Lakdawala's name from the section of the text that recognizes her assistance. The authors have consented to this revision.

RESEARCH ARTICLE

Ibuprofen reverts antifungal resistance on *Candida albicans* showing overexpression of CDR genes

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Candida albicans; ibuprofen; azoles resistance genes; antifungal resistance; real-time PCR.

Abstract

Several mechanisms may be associated with *Candida albicans* resistance to azoles. Ibuprofen was described as being able to revert resistance related to efflux activity in *Candida*. The aim of this study was to uncover the molecular base of antifungal resistance in *C. albicans* clinical strains that could be reverted by ibuprofen. Sixty-two clinical isolates and five control strains of *C. albicans* were studied: the azole susceptibility phenotype was determined according to the Clinical Laboratory for Standards Institute, M27-A2 protocol and minimal inhibitory concentration values were recalculated with ibuprofen (100 $\mu g\,mL^{-1}$); synergistic studies between fluconazole and FK506, a Cdr1p inhibitor, were performed using an agar disk diffusion assay and were compared with ibuprofen results. Gene expression was quantified by real-time PCR, with and without ibuprofen, regarding *CDR1*, *CDR2*, *MDR1*, encoding for efflux pumps, and *ERG11*, encoding for azole target protein. A correlation between susceptibility phenotype and resistance gene expression profiles was determined. Ibuprofen and FK506 showed a clear synergistic effect when combined with fluconazole. Resistant isolates reverting to susceptible after incubation with ibuprofen showed *CDR1* and *CDR2* overexpression especially of the latter. Conversely, strains that did not revert displayed a remarkable increase in *ERG11* expression along with CDR genes. Ibuprofen did not alter resistance gene expression significantly ($P > 0.05$), probably acting as a Cdrp blocker.

Introduction

Candida spp., especially *Candida albicans*, are common opportunistic yeasts causing mucocutaneous and systemic infections with high morbidity and mortality. Such infections are often associated with immune disorders, endocrine abnormalities and unrestricted use of large-spectrum antimicrobials being treated with antifungals such as azoles, the most widely used drugs (Carrillo-Munoz *et al.*, 2006).

Several different mechanisms may confer antifungal resistance in *C. albicans* clinical strains (White *et al.*, 1998; Sanglard & Odds, 2002); its molecular base is being documented gradually (de Micheli *et al.*, 2002; Silver *et al.*, 2004; Harry *et al.*, 2005; Coste *et al.*, 2006, 2007; Pasrija *et al.*, 2007). A common mechanism described involves the reduction of the antifungal intracellular concentration due to efflux pump overexpression encoded by *CDR1*, *CDR2* and *MDR1* genes (Fling *et al.*, 1991; Prasad *et al.*, 1995; Sanglard *et al.*, 1997), the former two related to azole cross-resistance, and the third associated with selective resistance to fluconazole. Cdr1p and Cdr2p are transmembrane proteins belonging to the ABC family, ATP dependent; the substrates for such proteins are not specific and include unrelated compounds such as steroids and lipids and antifungals such as azoles. However, its respective mode of action is not yet fully understood. Mdr1p belongs to the Major Facilitator protein family, which uses proton motive force to obtain energy. As described previously, a deletion in any of these genes confers hypersusceptibility to azoles (Coste *et al.*, 2004). Another resistance mechanism described involves point mutations in the *ERG11* gene leading to protein amino acidic sequence alterations, thus decreasing azole affinity for its molecular

target. The *ERG11* gene encodes for lanosterol 14α-demethylase (Marichal *et al.*, 1999), a cytochrome P450 enzyme involved in ergosterol biosynthesis, one of the most abundant lipids in fungal cell membranes. An increase in its expression might also result in protein overproduction that may overcome the azole concentration and its effects.

Previous studies showed that antifungal resistance in *Candida* spp. clinical strains could be reverted with modulators such as ibuprofen (Pina-Vaz *et al.*, 2005). Ibuprofen inhibited extrusion mechanisms, promoting the intracellular accumulation of the fluorescent probe FUN-1 or [H^3] – itraconazole in blastoconidia, as confirmed by flow cytometry and by cintilography, respectively (Pina-Vaz *et al.*, 2005). A similar effect was described with compounds such as FK506 (tacrolimus), a potent immunosuppressor agent and calcineurin inhibitor, showing a synergistic effect when combined with antineoplastic agents on tumour cells, decreasing or even suppressing drug resistance; it competes with cytotoxic drugs for a binding site on *P*-glycoprotein, being able to revert multidrug resistance by such cells (Arceci *et al.*, 1992). Reversion studies have also addressed efflux-resistant *C. albicans* strains and FK506 (Marchetti *et al.*, 2000; Sun *et al.*, 2008).

In order to characterize the resistant *C. albicans* strains reverting with ibuprofen and to assess whether such a drug might interfere with efflux pump activity, a molecular study was performed in order to quantify *CDR1*, *CDR2*, *MDR1* and *ERG11* gene expression in the presence and absence of ibuprofen. The elucidation of such mechanisms could help in the development of new therapeutic strategies, aimed at blockade of the efflux proteins and the subsequent reversion of antifungal resistance, or to new approaches in drug design, namely of compounds avoiding the extrusion of antifungal drugs.

Materials and methods

Strains

Sixty-two *C. albicans* clinical strains (42 resistant, R and 20 susceptible, S to azoles), isolated from the respiratory tract, vaginal fluid, urine and blood, were included. Five *C. albicans* strains with known different susceptibility phenotypes and with well-characterized gene expressions were also included as controls: strains 95–142 and 95–190 (both with *CDR1* and *CDR2* overexpression) and strain 12–99 (with *CDR1*, *CDR2*, *MDR1* and *ERG11* overexpression), all three R to azoles, and the S strain 2–76 (without gene overexpression associated with resistance), kindly gifted by Dr Theodore C. White, Seattle, WA (White *et al.*, 2002); *C. albicans* ATCC 90028 type strain, from American Type Culture Collection, as recommended by the susceptibility test reference protocol (CLSI, 2002). The strains were kept at −70 °C in brain/heart infusion (Difco) with 10% glycerol and subcultured twice in Sabouraud agar (Difco), before testing to assure purity of cultures.

Antifungals, drugs and stock solution preparation

Fluconazole (Pfizer) stock solution was prepared in sterile distilled water at a final concentration of 640 μg mL^{-1}; itraconazole (Jansen) and voriconazole (Pfizer) were prepared in dimethyl sulphoxide (DMSO) (Sigma) at a final concentration of 1600 μg mL^{-1}. FK506 (Sigma) and ibuprofen (Sigma) stock solutions were prepared in DMSO and sterile distilled water, respectively, both at a final concentration of 1 mg mL^{-1}. All stock solutions were stored at −70 °C, except for ibuprofen, which was kept at 4 °C until use.

Phenotypical analysis

Broth microdilution susceptibility testing method for azoles with and without ibuprofen

Minimal inhibitory concentrations (MIC) of fluconazole (Pfizer), itraconazole (Jansen) and voriconazole (Pfizer) were determined according to the protocol established by the Clinical Laboratory for Standards Institute (CLSI) (formerly NCCLS), M27-A2 (CLSI, 2002). Antifungal serial dilutions were prepared from the respective stock solutions. The visual readings were performed following 24 and 48 h of incubation at 37 °C; MIC was defined as the lowest concentration that produced a significant reduction (about 80%) in cellular growth in comparison with control cells that were not exposed to the antifungal, as recommended. The strains were classified as S, R or susceptible-dose dependent (S-DD) according to the CLSI protocol. For all strains, MIC values of the tested antifungals were redetermined in the presence of a subinhibitory concentration of ibuprofen (100 μg mL^{-1}) (Pina-Vaz *et al.*, 2005). Strains were clustered, according to azole MIC values in the presence of ibuprofen, into three distinct phenotypic groups: S, R_R (Resistant Reverting) and R_{NR} (Resistant Non-Reverting).

Agar disk diffusion assays with FK506

Control strains 95–190 and 95–142, both R to fluconazole but reverting to S when fluconazole was associated with ibuprofen (R_R), and two clinical strains, one R_R and one R_{NR} strain (did not revert to S in the presence of ibuprofen), were incubated overnight in YEPD liquid medium at 37 °C, 150 r.p.m. The inoculum was prepared after resuspending the overnight growth culture in sterile distilled water at 0.5 McFarland density standard at 530 nm wavelength (Densimat; Biomérieux, France), corresponding to a final

Table 1. Primer sequences used in real-time PCR for *CDR1*, *CDR2*, *MDR1*, *ERG11* and *ACT1* gene expression quantification in *Candida albicans* strains

Gene	GenBank accession no. (reference)	Primer concentration (μM)	Annealing temperature (°C)	Primers	Sequence
CDR1	X77589 (Prasad *et al.*, 1995)	0.5	58	Forward	5′-TGCCAAACAATCCAACAA-3′
				Reverse	5′-CGACGGATCACCTTTCATACGA-3′
CDR2	U63812 (Sanglard *et al.*, 1997)	0.5	58	Forward	5′-AAGGTTTTGATGCTACTGC-3′
				Reverse	5′-GTCGGACATGTGGCTCAAA-3′
MDR1	X53823 (Fling *et al.*, 1991)	0.7	55	Forward	5′-GTGTTGGCCCATTGGTTTTCAGTC-3′
				Reverse	5′-CCAAAGCAGTGGGGATTTGTAG-3′
ERG11	X13296 (Lai & Kirsch, 1989)	0.7	55	Forward	5′-GGTGGTCAACATACTTCTGCTTC-3′
				Reverse	5′-GTCAAATCATTCAAATCACCACCT-3′
ACT1	X16377 (Losberger & Ernst, 1989)	0.7	58	Forward	5′-AAGAATTGATTTGGCTGGTAGAGA-3′
				Reverse	5′-TGGCAGAAGATTGAGAAGAAGTTT-3′

concentration of $1–5 \times 10^6$ CFU mL^{-1}. The yeast suspensions were spread onto YEPD agar plates containing fluconazole at a supra-MIC value (128 μg mL^{-1}), and without fluconazole. Blank paper disks, 6 mm (BBL, Becton Dickinson France S.A.), were impregnated with an FK506 solution in serial concentrations (10-fold dilutions ranging from 100 to 0.1 μg mL^{-1}) and with its solvent (DMSO), allowed to dry for 2 h. For each tested strain, the disks were applied in the inoculated agar plates (after previous assessment of FK506 nontoxicity, upon yeast cells). A plate containing only fluconazole (no FK506 disk) was used as a control for cell viability in the presence of fluconazole supra-MIC values. The agar plates were incubated at 37 °C, and the results were registered after 24 and 48 h.

Gene expression analysis

Total RNA extraction from yeast cell cultures

All strains were incubated in Sabouraud broth (Difco) at 37 °C with continuous shaking, 150 r.p.m., until the mid-log phase. A mid-log phase culture of each strain was also incubated for 90 min, with ibuprofen 100 μg mL^{-1} (Pina-Vaz *et al.*, 2005). Total RNA was extracted using the hot acid phenol method, as described by Köhrer & Domdey (1991), and quantified in a biophotometer 6131 (Eppendorf®, Hamburg, Germany). To confirm RNA integrity, *c.* 200–300 ng of RNA solution was run in a 1% agarose gel (Sigma). RNA samples were kept at −70 °C for later use.

Reverse transcriptase (RT)-PCR

Two-step real-time PCR reactions were prepared in a total volume of 20 μL, which included: 50 ng of RNA sample, 0.03 ng μL^{-1} Random Primer (Invitrogen), 1 × transcriptase reverse enzyme Buffer (Invitrogen), 2 U μL^{-1} reverse transcriptase enzyme (Invitrogen), 0.1 M dithiothreitol (Invitrogen), 1.9 mM $MgCl_2$ (Fermentas), 0.5 mM dNTPs (Fermentas), 1 U μL^{-1} RNAsin enzyme (Promega) and RNAse-free water. Reactions were carried out in a GeneAmp PCR System 9600 (Perkin Elmer) with the following parameters: 10 min at 22 °C, 50 min at 42 °C and 10 min at 95 °C. The reaction mixtures were kept at –20 °C for later real-time PCR amplification.

Real-time PCR

For primer design, target gene sequences (*CDR1*, *CDR2*, *MDR1* and *ERG11*) and the housekeeping gene sequence (*ACT1*, used as a normalizing gene) were obtained from NCBI home page (http://www.ncbi.nlm.nih.gov). Primers (*Thermo*, listed in Table 1) were designed using the program PRIMER SELECT (DNAStar) and tested through the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). Real-time PCR reaction included: different primer concentrations, ranging from 0.5 to 0.7 μM (Table 1), SYBR Green 1x format (QuantiTect SYBR Green PCR Kit 200, Qiagen), 2 μL of cDNA from RT-PCR and RNAse-free water, in a 20-μL final reaction volume. Distinct $MgCl_2$ concentrations were used for each target gene (3 mM for *ACT1* and 1 mM for *CDR1*, *CDR2*, *MDR1* and *ERG11*). All reactions were performed in the Light Cycler 1.5 System (Roche®) and parameters (detailed in Table 1) were chosen according to the manufacturer's recommendations. To check for PCR product specificity, a melting curve was established (temperature ranging from 65 to 95 °C, for 1.5 min). In each assay, a standard curve was obtained, in duplicate, containing serial twofold dilutions from a known concentration of RNA transcribed to cDNA, to assess quantification and reaction efficiency. The results were analysed using the program LIGHTCYCLER software version 3.5 from Roche®. PCR amplification products were subjected to electrophoresis in a 1% agarose gel, stained with ethidium bromide and visualized with UV light. Relative gene expression was calculated as a ratio of each target gene relative to the *ACT1* gene

Table 2. Susceptibility profile in the presence and absence of ibuprofen and *CDR1*, *CDR2*, *MDR1* and *ERG11* basal gene expression, normalized with *ACT1* in *Candida albicans* control strains

	MIC		Gene expression level			
Control strains	Fluconazole ($\mu g\,mL^{-1}$)	Fluconazole + ibuprofen ($\mu g\,mL^{-1}$)	*CDR1*	*CDR2*	*MDR1*	*ERG11*
ATCC 90028	0.5 (S)	0.5 (S)	1.22 ± 0.07	0.18 ± 0.01	ND	0.93 ± 0.07
2–76	1 (S)	1 (S)	1.36 ± 0.09	1.07 ± 0.06	ND	0.44 ± 0.03
12–99	64 (R)	32 (S-DD)	37.37 ± 0.93	41.88 ± 1.23	1.169 ± 0.09	1.18 ± 0.11
95–190	128 (R)	1 (S)	88.35 ± 3.21	90.53 ± 2.43	0.04 ± 0.01	2.86 ± 0.13
95–142	128 (R)	1 (S)	4.19 ± 0.17	3.63 ± 0.17	0.02 ± 0.01	1.88 ± 0.09

S, susceptible; R, resistant; S-DD, susceptible-dose dependent.
ND, not detected (expression below detectable values).

Table 3. Fluconazole, voriconazole and itraconazole MIC for *Candida albicans* clinical strains ($n = 62$) and its respective susceptibility patterns

No. of strains/phenotype			
MIC fluconazole	MIC voriconazole	MIC itraconazole	MIC fluconazole + ibuprofen
20/S (0.25–8 $\mu g\,mL^{-1}$)	20/S (0.015–0.25 $\mu g\,mL^{-1}$)	20/S (0.125 $\mu g\,mL^{-1}$)	20/S (0.25–8 $\mu g\,mL^{-1}$
42/R (64–256 $\mu g\,mL^{-1}$)	42/R (8–16 $\mu g\,mL^{-1}$)	42/R (4–8 $\mu g\,mL^{-1}$)	40/S (0.5–8 $\mu g\,mL^{-1}$); 1/S-DD and1/R (16–64 $\mu g\,mL^{-1}$)

The MIC of fluconazole was determined in the presence of a subinhibitory concentration of ibuprofen (100 $\mu g\,mL^{-1}$). The breakpoints considered for the different azoles were: for fluconazole resistant (R) MIC $\geq$ 64 $\mu g\,mL^{-1}$, susceptible-dose dependent (S-DD) MIC between 16–32 $\mu g\,mL^{-1}$ and susceptible (S) MIC $\leq$ 8 $\mu g\,mL^{-1}$; for voriconazole, R MIC $\geq$ 4 $\mu g\,mL^{-1}$, S-DD MIC = 2 $\mu g\,mL^{-1}$ and S MIC $\leq$ 1 $\mu g\,mL^{-1}$; for itraconazole, R MIC $\geq$ 1 $\mu g\,mL^{-1}$, S-DD MIC between 0.25 and 0.5 $\mu g\,mL^{-1}$, S MIC $\leq$ 0.125 $\mu g\,mL^{-1}$; n, number of strains.

concentration, for each of the three sets of strains studied (S, R_R and R_{NR}).

Statistical analysis

Student's *t*-test was used to compare the gene expression among the different groups studied. A *P*-value < 0.05 was considered significant.

Results

Azole susceptibility in the presence or absence of ibuprofen

The results obtained with the control strains are detailed in Table 2. Only the R strains 95–190 and 95–142, displaying *CDR* overexpression, showed a reduction in azole MIC values following incubation with ibuprofen; S strains and R strains overexpressing all the screened resistance genes (*CDR*, *MDR1* and *ERG11*) showed minimal changes in azole MIC values following incubation with ibuprofen.

The azole susceptibility pattern of the clinical isolates determined in the presence or absence of ibuprofen is detailed in Table 3. In the presence of ibuprofen, the MIC values did not change in S strains, while most R strains, which presented cross-resistance to all the assayed azoles, reverted to S, therefore being designated as R_R; one R strain maintained the R profile while another became S-DD, being designated as R_{NR}.

Synergistic effect between fluconazole and FK506

For strains classified as R_R, a synergistic effect between fluconazole and FK506 was detected using the agar diffusion method (Fig. 1); in control plates with no fluconazole added to the medium, neither FK506 nor its solvent was able to inhibit *Candida* growth (Fig. 1, left panel), which shows that such a compound does not exhibit antifungal activity. Regarding the control strain 95–142, a clear synergistic effect was already observed at the lowest FK506 concentration assayed (Fig. 1, right panel) while with the clinical strain the same effect was observed only at higher concentrations (right panel). Conversely, with R_{NR} strains, complete growth inhibition was not achieved under similar experimental conditions (Fig. 1).

CDR1, *CDR2*, *MDR1* and *ERG11* gene expression quantification

The results for the control strains were in agreement with previously published data (White *et al.*, 2002): S control strains showed low expression for target genes while R strains displayed an evident increase in *CDR1* and *CDR2* gene expression; strain 12–99 was the single strain

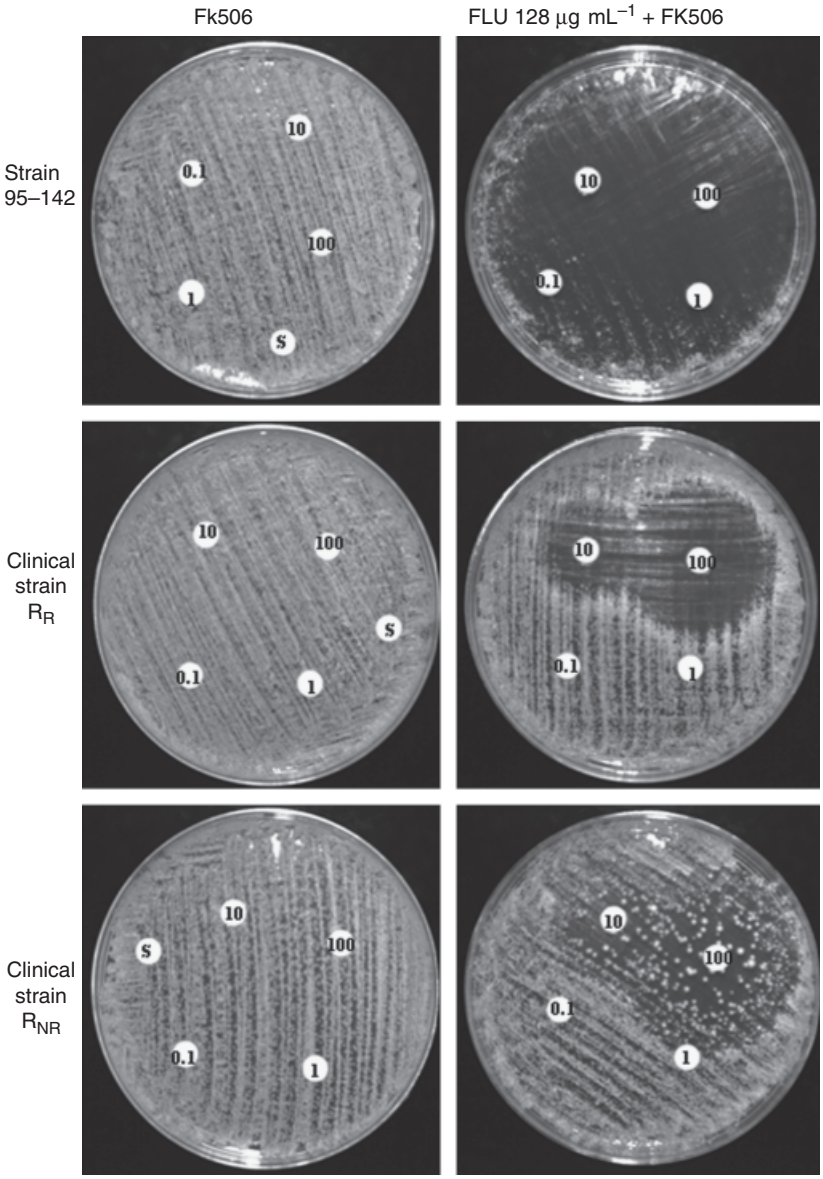

Fig. 1. Disk diffusion assays with three resistant *Candida albicans* strains in the presence of FK506. Control strain 95–142 (R_R) and two clinical strains (one R_R and one R_{NR}) grown on YEPD medium and paper disks impregnated with serial dilutions of FK506 (0.1, 1.0, 10 and 100 μg mL^{-1}) and DMSO (S-solvent), in the presence of fluconazole (FLU 128 μg mL^{-1}) (right panel) or in its absence (left panel).

presenting *MDR1* expression. Regarding *ERG11*, all R strains displayed overexpression relative to S (Table 2). Interestingly, we have found higher *ERG11* expression in strains 95–190 and 95–142 than in the 12–99 strain; most certainly, the gene in these strains is not associated with resistance as will be discussed later.

The mean values for target gene expression for the three considered sets of clinical strains (S, R_R and R_{NR}) are shown in Fig. 2. *CDR1* expression was higher in R_{NR} and R_R strains compared with S. The difference was significant between S and R_{NR} strains ($P = 0.008$) but not between R_R and S strains or between the two groups of R strains (R_R and R_{NR}) ($P > 0.05$). In terms of *CDR2* expression, significant differences were found on comparing S with R_R ($P = 0.002$) or with R_{NR} ($P = 0.044$) strains; no significant difference was found between the two R groups. No *MDR1* gene expression was detected among clinical isolates (data not shown). *ERG11* gene expression was similar between S strains and R_R ($P > 0.05$), while significant differences were found between R_{NR} and S ($P = 0.005$) and between R_R and R_{NR} strains ($P = 0.004$).

Incubation with ibuprofen, under the described experimental conditions, did not result in significant changes of resistance gene expression ($P > 0.5$).

Discussion

Resistance among *C. albicans* represents a serious therapeutic problem. Different mechanisms may be expressed, allowing the organisms to elude the antifungal effect (Sanglard & Odds, 2002).

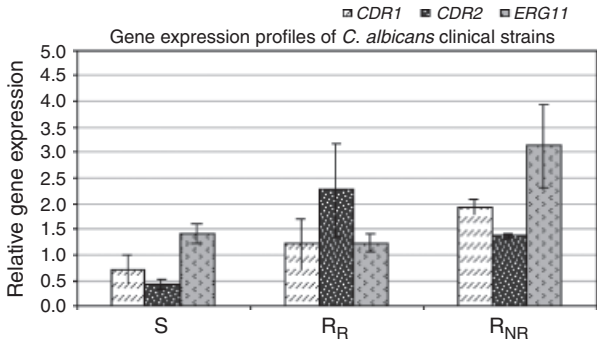

Fig. 2. Mean target gene expression levels of *Candida albicans* clinical isolates. *CDR1*, *CDR2*, *MDR1* and *ERG11* gene expression levels were quantified and normalized relative to the housekeeping gene, *ACT1*, in clinical isolates grouped according to their susceptibility phenotype: susceptible (S), resistant strains that reverted with ibuprofen (R_R) and resistant strains that did not revert with ibuprofen (R_{NR}).

We have studied a high number of *C. albicans* clinical resistant strains regarding resistance target gene expression. The *CDR1* and *CDR2* gene overexpression associated with an efflux-related resistance mechanism appears to be quite prevalent, which may indicate the ibuprofen treatment strategy as quite promising.

Previous studies conducted by our team described ibuprofen as a potential efflux blocker; FK506 was also described as being able to revert *C. albicans* azole resistance due to a synergistic effect with azoles (Maesaki *et al.*, 1998; Onyewu *et al.*, 2003; Sun *et al.*, 2008). This effect seems to result from efflux pump blockade, especially of Cdr1p (Schuetzer-Muehlbauer *et al.*, 2003). To corroborate our results with ibuprofen and its possible involvement in efflux blockade, we performed disk diffusion assays with FK506 in a small group of strains; a synergistic effect similar to ibuprofen was obtained. Control strain 95–142 revealed much lower *CDR1* and *CDR2* gene expression levels than control strain 95–190 (Table 2), which could explain the results obtained in the disk diffusion assays: in the former, reversion of resistance occurred with all FK506 concentrations tested, suggesting that efflux is much less efficient on such a strain. On the other hand, in R_R strains *CDR1* and *CDR2*, high gene expression levels were overcome in the presence of FK506; ibuprofen yielded an effect similar to FK506, eventually blocking efflux pumps. R_{NR} strains might express other distinct resistance mechanisms.

MDR1 gene expression was not detected among clinical R strains (R_R and R_{NR}); this gene is invariably associated with selective resistance to fluconazole (White *et al.*, 2002) and does not seem to be a common or a relevant antifungal resistance mechanism among clinical isolates. Nevertheless, two facts should be highlighted: the clinical isolates showed azole cross-resistance, but not specific fluconazole resistance, and strains 12–99, used as a positive control for *MDR1* quantification, displayed *MDR1* overexpression when compared with S control strains 2–76 (White *et al.*, 2002). It is possible that in such strains, Cdr1p and Cdr2p activity overcome Mdr1p efflux activity; on the other hand, *CDR1* and *CDR2* encoding mRNA has a longer half-life than *MDR1* encoding mRNA (Lyons & White, 2000), thus being more easily detected in gene expression studies.

The *ERG11* gene is not always associated with antifungal resistance (White *et al.*, 2002), thus being expressed at relatively high levels in both S and R (R_R and R_{NR}) strains. Apart from a high *ERG11* gene expression, R_{NR} strains also revealed *CDR* gene overexpression, displaying most probably multiple resistance mechanisms. Ibuprofen was unable to revert the resistance of such strains, which corroborates our hypothesis of ibuprofen activity being related to efflux pump activity inhibition. In accordance with this assumption, control strain, 12–99, possessing multiple resistance mechanisms including *ERG11* gene overexpression associated with resistance, revealed the same phenotypical and genotypical behaviour as R_{NR} strains (Redding *et al.*, 1994; White, 1997; White *et al.*, 2002). In addition, the fact that incubation with ibuprofen did not impair resistance gene expression reinforces the concept of its direct effect on efflux pumps, most probably inducing its physical blockade.

Although the clinical strains were phenotypically homogeneous within the same group, when target gene expression levels were determined, a high intragroup variability in gene expression profiles was observed, thus resulting in high SD values. This variation might result from the fact that some clinical isolates might have been previously exposed to antifungals *in vivo*. Antifungal resistance acquisition over time has already been described (Franz *et al.*, 1998; Cowen *et al.*, 2000; Karababa *et al.*, 2004) and what occurs at a molecular level is being progressively elucidated (Silver *et al.*, 2004; Coste *et al.*, 2007; Morschhauser *et al.*, 2007).

In conclusion, clinical R strains revealing *ERG11* overexpression and eventually presenting point mutations will not respond to efflux blockers, such as ibuprofen, even when azole resistance also involves efflux mechanisms. Conversely, whenever efflux genes are the most expressed resistance genes, reversion by ibuprofen will be feasible and of considerable future therapeutic interest. The uncovering of molecular bases of *Candida* resistance mechanisms, apart from representing a major challenge for molecular biologists, is of major interest for clinicians who seek new and more targeted therapeutic strategies, aimed at the reversion of resistance by clinical fungal pathogens such as *C. albicans*.

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RESEARCH ARTICLE

Dynamics of *in vitro* acquisition of resistance by *Candida parapsilosis* to different azoles

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Candida parapsilosis; antifungal resistance; azoles; inducible resistance.

Abstract

Candida parapsilosis is a common isolate from clinical fungal infectious episodes. Resistance of *C. parapsilosis* to azoles has been increasingly reported. To analyse the development of resistance in *C. parapsilosis*, four azole-susceptible clinical strains and one American Type Culture Collection type strain were cultured in the presence of fluconazole, voriconazole and posaconazole at different concentrations. The isolates developed variable degrees of azole resistance according to the antifungal used. Fluconazole was the fastest inducer while posaconazole was the slowest. Fluconazole and voriconazole induced resistance to themselves and each other, but not to posaconazole. Posaconazole induced resistance to all azoles. Developed resistance was stable; it could be confirmed after 30 days of subculture in drug-free medium. Azole-resistant isolates revealed a homogeneous population structure; the role of azole transporter efflux pumps was minor after evaluation by microdilution and cytometric assays with efflux pump blockers (verapamil, ibuprofen and carbonyl cyanide 3-chloro-phenylhydrazone). We conclude that the rapid development of azole resistance occurs by a mechanism that might involve mutation of genes responsible for ergosterol biosynthesis pathway, stressed by exposure to antifungals.

Introduction

During the last decade *Candida* species have emerged as major opportunistic pathogens, mainly due to the increase in the number of immunocompromised patients (Pfaller & Diekema, 2007). Among *Candida* species causing nosocomial infections, the opportunistic yeast *Candida parapsilosis* is at present the second or third most common blood culture fungal isolate in Europe, Canada and Latin America, outranking *Candida albicans* in some European hospitals (Pfaller & Diekema, 2007).

Although sometimes considered a member of the indigenous microbial population in healthy individuals, *C. parapsilosis* can be also recovered from distinct environmental sources and from the hands of health care workers, thus suggesting its role as a potential route for nosocomial transmission (Levin *et al.*, 1998; Kojic & Darouiche, 2004).

Unfortunately, fungal pathogens acquire resistance to azoles, particularly after prolonged exposure, as is the case with its prophylactic overuse. The widespread use of antifungal molecules, especially fluconazole, has selected *Candida* species with easily inducible resistance, such as *Candida glabrata* (Wingard *et al.*, 1993) and *Candida tropicalis* (Barchiesi *et al.*, 2000) or species that show intrinsic resistance, such as *Candida krusei* (Wingard *et al.*, 1991). *Candida albicans* resistance to fluconazole was promoted following incubation with subinhibitory concentrations of the drug (Marr *et al.*, 2001). In the case of an antifungal drug being more prone to induce *in vitro* resistance in comparison with others, this might represent a hypothetical drawback for its use *in vivo*, at least for prophylactic treatment.

Three distinct mechanisms of azole resistance have been described so far in *C. albicans*: (1) failure to accumulate the drug intracellularly, which may be caused by the lack of drug

penetration due to changes in membrane lipids or sterols (Hitchcock *et al.*, 1986) or (2) by active efflux of drugs resulting from overexpression of genes *CDR1*, *CDR2* and *MDR1* (Albertson *et al.*, 1996; Sanglard *et al.*, 1996; White, 1997; Marr *et al.*, 1998); and (3) increased production of the azole target enzyme and point mutations in genes that codify for this enzyme, the products of which have reduced affinity to azoles (Sanglard *et al.*, 1998). Lanosterol-14α-demethylase is a common target for all azole antifungals. This enzyme belongs biochemically to the group of cytochrome P450 enzymes and is involved in the synthesis of ergosterol, which is a major and essential lipid constituent of the cell membrane of fungi (Akins, 2005). All azoles, including posaconazole (Munayyer *et al.*, 2003; Hof, 2006), inhibit the production of ergosterol, causing a depletion of this compound. Mutations in other enzymes involved in the synthesis of ergosterol may also contribute to azole resistance.

Although several resistance mechanisms may operate in fungal pathogens, efflux-mediated drug tolerance is the major factor responsible for clinical resistance (Kontoyiannis & Lewis, 2002; Sanglard & Odds, 2002; White *et al.*, 2002). Previous studies demonstrated that resistance related to overexpression of efflux pumps can be reverted by modulators, particularly verapamil or sex hormones (Ford & Hait, 1990) and more recently by ibuprofen (Pina-Vaz *et al.*, 2005). Inhibition of the efflux pumps that are H^+-dependent in yeasts by carbonyl cyanide 3-chlorophenylhydrazone (CCCP) has also been demonstrated (Prudêncio *et al.*, 2000; Guinea *et al.*, 2006).

Surprisingly, very few data are yet available regarding the mechanisms of azole resistance in *C. parapsilosis*.

The initial aim of our study was to assess and characterize the *in vitro* induction of resistance by distinct azole antifungals at different concentrations in four blood culture isolates and one American Type Culture Collection (ATCC) type strain of *C. parapsilosis*. Additional aims of this study were to elucidate whether such induced resistance is stable and to assess the involvement of efflux pumps.

Materials and methods

Strains

Four blood culture isolates of *C. parapsilosis* (BC014, BC011, BC237 and BC190) isolated from patients admitted to Hospital S. João in Porto, Portugal, were used in this study. They were characterized by Vitek YBC identification cards (BioMérieux, Paris, France). Until testing, the strains were stored in Brain–Heart broth (Difco) with 5% glycerol at −70 °C. For each experiment, each strain was subcultured twice on Sabouraud agar (Difco) at 35 °C for 48 h. *Candida parapsilosis* type strain ATCC 22019 was included. To study the role of efflux pumps on antifungal-induced resistance, two *C. albicans* strains with well-characterized mechanisms of resistance were also included as controls: *C. albicans* strain 95-68, with overexpression of *CDR1* and *CDR2* genes (ATP-dependent efflux pumps), and *C. albicans* strain 2-76, showing lower levels of resistance gene expression (a kind gift from Dr Ted White).

Chemicals and culture media

Standard powders of fluconazole (Pfizer, Groton, CT), voriconazole (Pfizer, New York, NY), posaconazole (Schering-Plough, Kenilworth, NJ), amphotericin B (AMB; Bristol Myers Squibb, New York, NY) and caspofungin (CAS; Merck, Rahway, NJ) were obtained from the respective manufacturers. A stock solution of fluconazole and CAS were prepared in distilled water; voriconazole, posaconazole and AMB were prepared with dimethyl sulfoxide. Antifungal drugs were diluted afterwards with Roswell Park Memorial Institute 1640 medium (RPMI 1640; Sigma, St. Louis, MO) buffered to pH 7.0 with 0.165 M morpholine propanesulfonic acid buffer (MOPS; Sigma) and stored at −70 °C until use. Verapamil, ibuprofen and CCCP were purchased from Sigma. The culture medium used for induction assays of resistance and evaluation of its stability was RPMI 1640 with 0.165 M MOPS, pH 7.0. Rhodamine 6G (Rh-6G) used in flow cytometric assays was purchased from Sigma.

Strategy for induction of antifungal resistance and for assessing its stability

A single, randomly selected colony from each *C. parapsilosis* strain was incubated in 10 mL of RPMI 1640 overnight in a rotating drum at 150 r.p.m., 35 °C. An aliquot of this culture, containing 10^6 blastoconidia, was transferred to different vials, each containing 10 mL of culture medium with or without an antifungal drug (fluconazole, voriconazole or posaconazole), and incubated overnight as described above. The following day, aliquots from each culture containing 10^6 blastoconidia were again transferred into fresh medium containing the same antifungal and reincubated as described. Each day, for the 30 days of the assay, a 1-mL aliquot from each subculture was mixed with 0.5 mL of 50% glycerol and frozen at −70 °C for later testing. In this type of experiment two different approaches were taken: (1) incubation for 30 days with constant concentrations of fluconazole ($16\,\mu g\,mL^{-1}$), voriconazole ($2\,\mu g\,mL^{-1}$) or posaconazole ($1\,\mu g\,mL^{-1}$); these concentrations correspond to therapeutic serum levels obtained during antifungal treatment (Azanza *et al.*, 2007) and (2) incubation with fluconazole, voriconazole and posaconazole at twice the concentration of the minimal inhibitory concentration (MIC) value, for 2 weeks, and afterwards at four times the concentration until the 30th day.

To assess resistance stability, the resistant isolates obtained were subcultured daily in the absence of the drug for 30 days. A single colony from each isolate was incubated in 10 mL drug-free RPMI 1640 at 35 °C, 150 r.p.m., The following day, aliquots were transferred into fresh medium. At each subculture, a 1-mL aliquot of the suspension was mixed with 0.5 mL of 50% glycerol, and frozen at −70 °C for later testing.

Antifungal susceptibility testing

The MIC values of each antifungal drug were determined according to the Clinical Laboratory Standards Institute (CLSI, 2008) M27-A3 protocol. MIC was registered after 24 and 48 h. Interpretative criteria for fluconazole and voriconazole were those of the CLSI: for fluconazole: susceptible (S)-MIC $\leq 8\,\mu g\,mL^{-1}$, susceptible-dose dependent (S-DD)-MIC 16–32 $\mu g\,mL^{-1}$, and resistance (R)-MIC $\geq 64\,\mu g\,mL^{-1}$; for voriconazole, S-MIC $\leq 1\,\mu g\,mL^{-1}$ and R-MIC $\geq 4\,\mu g\,mL^{-1}$. The interpretive criteria for CAS were those recently assigned by the CLSI (June 2007): S $\leq 2\,\mu g\,mL^{-1}$. Although susceptibility breakpoints have not yet been established for posaconazole and AMB, strains inhibited by $\leq 1\,\mu g\,mL^{-1}$ of each were considered to be susceptible (Pfaller *et al.*, 2001, 2003). Every 5 days of incubation, with or without antifungal, MIC values were redetermined for the five antifungals tested. *Candida parapsilosis* type strain ATCC 22019 was used in each testing assay, as recommended.

Population analysis

This assay was performed as described previously by Marr *et al.* (2001). A single colony of the initially susceptible and of the final resistant isolates were cultured overnight in YEPD broth (1% yeast extract, 2% peptone, 2% glucose) at 35 °C; a suspension containing 10^3 blastoconidia mL^{-1} was prepared in RPMI 1640 and plated in Sabouraud agar with and without fluconazole (1, 4, 16 and 64 $\mu g\,mL^{-1}$), voriconazole (0.125, 0.5, 2 and 8 $\mu g\,mL^{-1}$) and posaconazole (0.25, 1, 4 and 16 $\mu g\,mL^{-1}$). Growth was quantified after 48 h of incubation at 35 °C. The number of colonies growing in the presence of the drug at each concentration, relative to the number growing in its absence, was calculated and plotted.

Study of efflux pumps

The role of efflux pumps in the resistance mechanism developed was evaluated using two different approaches:
(1) Antifungal MIC values were determined according to the CLSI M27-A3 protocol (CLSI, 2008) in the presence of different drugs described as efflux blockers: verapamil 100 μM, ibuprofen 100 $mg\,L^{-1}$ (Pina-Vaz *et al.*, 2005) and CCCP 0.5 $\mu g\,mL^{-1}$ (Guinea *et al.*, 2006).
(2) Flow cytometric analysis using Rh-6G, a specific substrate of efflux pumps was performed as described previously (Sanglard *et al.*, 1999; Posteraro *et al.*, 2003), to compare the induced resistant strains with susceptible strains (incubated in drug-free medium) and the induced resistant strains with and without incubation for 90 min with verapamil 100 μM, as described by Pina-Vaz *et al.* (2005). Briefly, yeast cells were grown to logarithmic phase in 5 mL of YEPD medium at 35 °C under constant agitation. Labelling of cells was conducted in 1 mL of YEPD, containing 10^7 blastoconidia and 10 μM Rh-6G for 30 min at 35 °C, after which the reaction was stopped by placing the tubes on ice. The mixture was then diluted 40-fold with cold sterile 0.1 M phosphate-buffered saline (Sigma) at pH 7.0 and analysed using flow cytometry in a FACSCalibur (BD Biosciences, Sydney, Australia) equipped with a 15-nm argon laser. From each yeast suspension, 30 000–50 000 blastoconidia were analysed; cell fluorescence was determined at FL1 (515 nm). All experiments were performed in duplicate.

Results

Development of resistance

Table 1 shows the MIC values determined for fluconazole, voriconazole and posaconazole before and after induction with different azoles for all tested strains. After exposure to therapeutic serum concentrations of the different azoles, distinct patterns of resistance were observed. Following 15 days of antifungal exposure, only the fluconazole susceptibility phenotype changed. This finding was observed with most tested strains exposed to fluconazole and with a few strains exposed to voriconazole; following posaconazole exposure, only minor MIC variations were registered after this short period of time. Fluconazole and voriconazole increased MIC values to themselves and to each other, the susceptibility phenotype changing to R after 30 days, but not to posaconazole, which maintained the S phenotype. Posaconazole induced resistance to all azoles only at the very end of incubation time. Therefore, the activity of fluconazole was the most impaired and it was the fastest inducer of resistance.

In a different strategy to compare the ability of the same azole drugs to induce resistance, incubation with MIC values two- and fourfold that of the antifungals were assayed. A representative example (type strain ATCC 22019) of the development of resistance in such instances is detailed in Fig. 1. The lower concentration of fluconazole was sufficient to induce resistance to itself and to voriconazole but not to posaconazole; the exposure of the yeast cells to the lower concentration of voriconazole only changed the fluconazole phenotype (S strain turned to S-DD), as only minor variations on MIC values were observed with

Table 1. MIC values (μg mL^{-1}) and phenotypes (Phen) of *Candida parapsilosis* type strain (ATCC 22019) and BC014, BC011, BC237 and BC190 clinical strains before and after induction with fluconazole (16 μg mL^{-1}), voriconazole (2 μg mL^{-1}) and posaconazole (1 μg mL^{-1})

		MIC/Phen								
		Fluconazole			Voriconazole			Posaconazole		
Induction with	Strains	Day 0	Day 15	Day 30	Day 0	Day 15	Day 30	Day 0	Day 15	Day 30
Fluconazole	ATCC	0.5/S	16/S-DD	64/R	0.015/S	0.25/S	2/S-DD	0.06/S	0.06/S	0.125/S
	BC014	1.0/S	16/S-DD	128/R	0.03/S	0.5/S	4/R	0.03/S	0.25/S	0.5/S
	BC011	1.0/S	8/S	128/R	0.03/S	0.25/S	2/S-DD	0.03/S	0.125/S	0.5/S
	BC237	1.0/S	32/S-DD	128/R	0.03/S	0.25/S	4/R	0.06/S	0.125/S	0.5/S
	BC190	0.5/S	16/S-DD	128/R	0.03/S	0.125/S	4/R	0.06/S	0.125/S	0.5/S
Voriconazole	ATCC	0.5/S	16/S-DD	64/R	0.015/S	0.06/S	2/R	0.06/S	0.06/S	0.125/S
	BC014	1.0/S	32/S-DD	128/R	0.03/S	1/S	4/R	0.03/S	0.125/S	0.5/S
	BC011	1.0/S	4/S	64/R	0.03/S	0.25/S	2/S-DD	0.03/S	0.125/S	0.5/S
	BC237	1.0/S	4/S	64/R	0.03/S	0.125/S	2/S-DD	0.06/S	0.125/S	0.5/S
	BC190	0.5/S	2/S	64/R	0.03/S	0.125/S	4/R	0.06/S	0.125/S	0.5/S
Posaconazole	ATCC	0.5/S	1/S	64/R	0.015/S	0.03/S	8/R	0.06/S	1/S	16/R
	BC014	1.0/S	1/S	128/R	0.03/S	0.03/S	16/R	0.03/S	0.125/S	32/R
	BC011	1.0/S	1/S	128/R	0.03/S	0.03/S	16/R	0.03/S	0.06/S	32/R
	BC237	1.0/S	1/S	64/R	0.03/S	0.03/S	2/S-DD	0.06/S	0.06/S	8/R
	BC190	0.5/S	1/S	128/R	0.03/S	0.06/S	16/R	0.06/S	0.125/S	32/R

the other tested azoles. Posaconazole had the least effect on the MIC values of all azoles in such experimental conditions.

Incubation with these three azoles, independently on the tested concentration, did not induce resistance to AMB and CAS.

Regarding the strains successively incubated without antifungals, the MIC values of the five antifungals tested remained constant throughout the study period.

Stability of azole resistance *in vitro*

Regarding the stability of the developed resistance by resistant organisms subcultured in drug-free medium for 30 days, the resistance pattern remained stable; no decreases greater than one dilution were observed in MIC values of all azoles tested.

Analysis of cellular populations

To determine whether the induced azole resistance in *C. parapsilosis* isolates was associated with selection of a resistant subpopulation, analysis of resistance within the cellular population was performed with susceptible and induced resistance strains. Population analysis clearly demonstrated the existence of a homogeneous population both for the resistant-induced strains and for the susceptibility strain. A representative example of these studies is shown for BC014 strain in Fig. 2.

Activity of efflux pumps

Effect of efflux blockers on MIC value of azoles

After incubation with ibuprofen, verapamil and CCCP, a decrease of MIC values of no more than one dilution was registered, and the resistance pattern remained stable. Regarding the control strains, *C. albicans* 95-68 in the presence of verapamil and ibuprofen, MIC values of fluconazole, voriconazole and posaconazole decreased drastically; as expected for strain *C. albicans* 2-76, the MIC values of the three antifungals did not change.

Rh-6G staining

The steady state regarding Rh-6G fluorescence of the susceptible phenotype (strains incubated in drug-free medium) and of resistant phenotypes obtained after 30 days of antifungal exposure (fluconazole 16 μg mL^{-1}, voriconazole 2 μg mL^{-1} and posaconazole 1 μg mL^{-1}) were compared. Similar intensity of fluorescence was obtained with induced resistant strains and susceptible strains (Fig. 3a). This intensity did not increase in the presence of the efflux blocker verapamil (data not shown). *Candida albicans* strain 95-68, with overexpression of *CDR1* and *CDR2* resistance genes, behaved as expected, showing an increase in Rh-6G staining when incubated with verapamil (Fig. 3b), whereas *C. albicans* strain 2-76 did not (data not shown), which validates the assays.

Discussion

Mechanisms of azole resistance due to antifungal exposure have been extensively investigated in clinical strains of *C. albicans* (Sanglard *et al.*, 1995, 1996, 1998; White, 1997; Franz *et al.*, 1998). Other studies have also elucidated the mechanisms of azole resistance in *C. glabrata*, *C. krusei* and *C. tropicalis* (Marichal *et al.*, 1997; Barchiesi *et al.*, 1998; Orozco

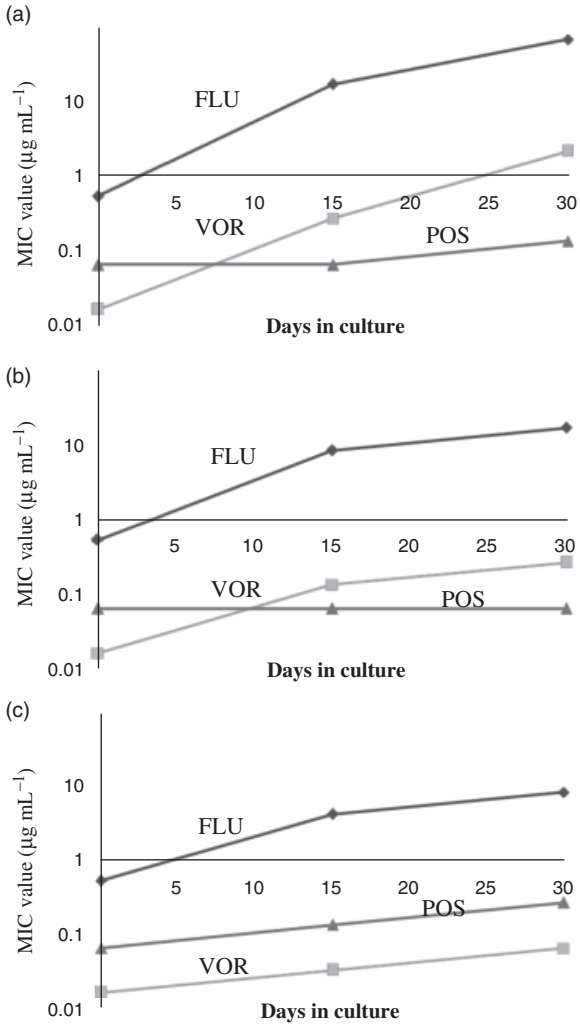

Fig. 1. Variation of fluconazole (FLU), voriconazole (VOR) and posaconazole (POS) MIC values for *Candida parapsilosis* type strain (ATCC 22019) cultured in medium containing two- and fourfold MIC values of FLU (a), VOR (b) and POS (c) for 30 days. —◆—, MIC of FLU; —■—, MIC of VOR; —▲—, MIC of POS.

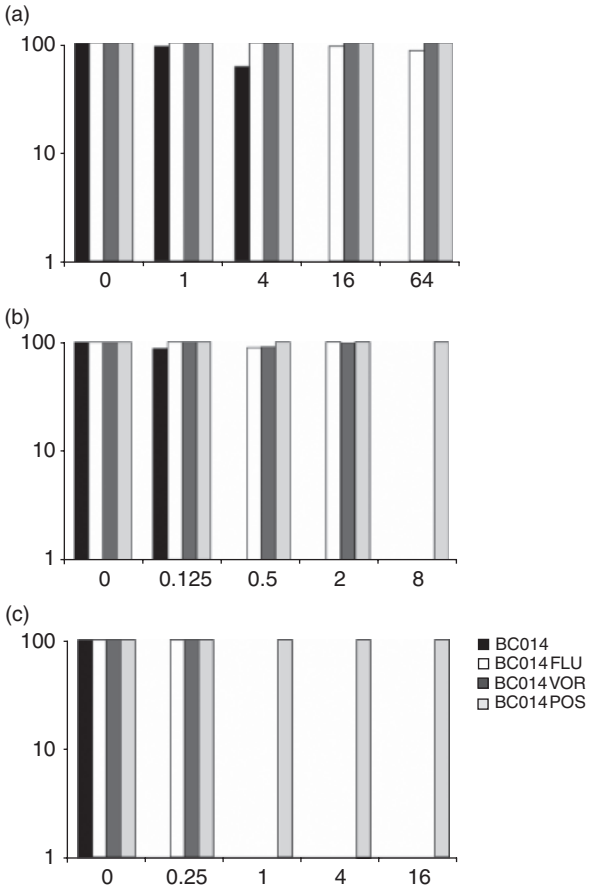

Fig. 2. Population analysis (a representative example) of a susceptible (BC014) and the respective resistant-induced strains following 30 days of exposure to azoles (BC014 FLU, BC014 VOR and BC014 POS). The percentage of resistant cells (*y*-axis, log scale) is defined as the ratio of colonies with growth on plates containing the indicated concentration of fluconazole (FLU) (a), voriconazole (VOR) (b) and posaconazole (POS) (c) relative to growth in the absence of drug.

et al., 1998). However, very few data are yet available regarding azole resistance in *C. parapsilosis*. This *Candida* species is an opportunistic yeast responsible for nosocomial infections, especially in immunocompromised or debilitated patients.

Resistance to fluconazole among *Candida* spp. has been reported to emerge whenever this drug is used extensively (Richardson, 2005). The results of our study stress several relevant characteristics of the *in vitro* acquisition of resistance by *C. parapsilosis* after prolonged exposure to antifungals. When incubated with a therapeutic fluconazole concentration (aiming to reproduce *in vivo* antifungal treatment), azole resistance quickly developed; similar results were obtained with a lower concentration of the same antifungal, showing that of the three azoles tested, fluconazole is an easy and fast inducer of resistance. The two different approaches used for induction of resistance corroborate this conclusion. This finding underlines the need to review therapeutic protocols. Both voriconazole and posaconazole induce resistance at concentrations of 2 and 1 μg mL^{-1}, respectively, but at lower concentrations the susceptibility pattern did not change drastically. As expected, incubation with any of the three azoles did not induce resistance to AMB and CAS, as azole targets are distinct and selective.

Our data strongly suggest that the dynamics of *in vitro* development of azole resistance in *C. parapsilosis* is quite different from that described for *C. tropicalis* and *C. albicans* (Calvet *et al.*, 1997; Barchiesi *et al.*, 2000; Marr *et al.*, 2001). For *C. albicans*, the time required to develop fluconazole resistance is longer and it could be fully reverted to baseline when the antifungal exposure stopped. *Candida albicans* isolates that became resistant showed an increase of mRNA

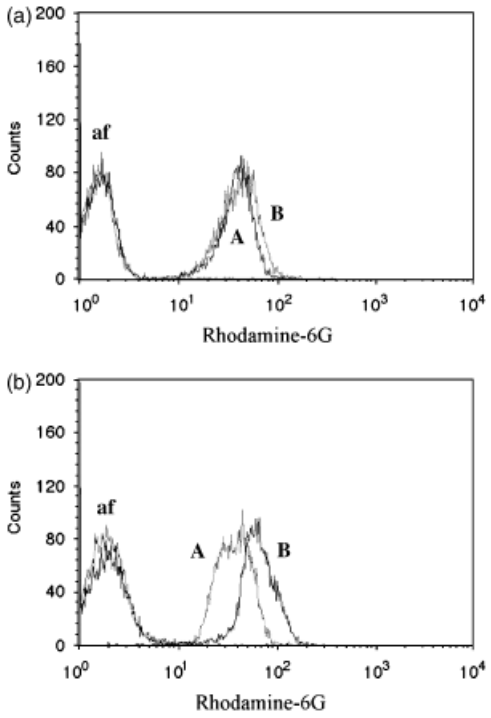

Fig. 3. Representative example of flow cytometric analysis of yeast blastoconidia after Rh-6G staining, an efflux pump substrate. (a) Histogram obtained by flow cytometric analysis at FL1 (515 nm) of BC014-susceptible strain incubated in drug-free medium (A) and BC014-resistant strain after incubation during 30 days with 16 μg mL^{-1} of fluconazole (B). (b) Histogram obtained by flow cytometric analysis at FL1 (515 nm) of resistant *Candida albicans* 95-68 strain (overexpression of genes that codify to efflux pumps) without (A) and with (B) the efflux blocker verapamil. af, autofluorescence of yeast blastoconidia.

specific for a *CDR* ATP-binding cassette transporter efflux pump (Marr *et al.*, 1998, 2001). The *in vitro* induction of resistance in *C. tropicalis* has been documented with associated increased expression of *CDR1* and *MDR1* genes (Barchiesi *et al.*, 2000), coding for a specific fluconazole transporter.

Distinct antifungal resistance mechanisms have been described among *Candida* spp., active efflux being the most relevant mechanism of azole resistance; its molecular base is relatively well known for *C. albicans* (Sanglard & Odds, 2002). Overexpression of these efflux proteins confers resistance to most azoles but in the same degree. Whereas ketoconazole, fluconazole, itraconazole and voriconazole are transported readily, posaconazole is transported to a much lesser extent (Chau *et al.*, 2004). Apparently, this compound is not the ideal substrate for these transporters.

If the underlying resistance mechanism induced after antifungal exposure were overexpression of efflux pumps, we would expect that in the presence of both types of efflux blockers MIC values would show a decrease and Rh-6G staining in resistant cells exposed to antifungal would be lower when compared with nonexposed cells. According to these results, the efflux seems to play a minor role on the resistance induced; however, the hypothesis of the existence of specific *C. parapsilosis* efflux pumps blocked by different modulators or with fluorescence substrate other than Rh-6G cannot be excluded.

In this study, the hypothesis that induced resistance in *C. parapsilosis* may be due to mutation in genes that codify enzymes of the ergosterol biosynthesis pathway gains considerable strength. This mutation is probably induced by the antifungal and does not result from selection of an initial resistant clone. This last hypothesis was not supported by the results from our population studies, as both the susceptible population (without antifungal exposure) as well as the resistant population, obtained following induction, were shown to be homogeneous.

The azoles bind in the vicinity of the haeme group of lanosterol-14α-demethylase. Although there are silent mutations scattered throughout the gene, at least a dozen different mutations in the active region result in a lower affinity for azoles, resulting in a greater influence on the antimicrobial activity of fluconazole and voriconazole than of posaconazole and itraconazole (Levin *et al.*, 1998). Moreover, the latter two drugs are able to bind to an additional domain of the enzyme by means of their long side chain (Li *et al.*, 2004; Akins, 2005), meaning that these two antifungals can still inhibit the target at a point when fluconazole and voriconazole are no longer active (Sabatelli *et al.*, 2006). Accordingly, such mutation should result in a lower impact on the susceptibility profile to posaconazole than to fluconazole or voriconazole. Because of the two different binding sites of posaconazole, the occurrence of large-spectrum cross-resistance is highly unlikely.

Different assumptions support the occurrence of drug-induced mutation during the described assays: the rapidity of development of the resistant phenotype; the fact that the three azoles are affected, although to a different extent; the insignificant role of efflux; and the stability of resistance following prolonged absence of drug.

Spontaneous mutations are very infrequent, occurring at frequencies of *c.* 10^{-6}–10^{-8} per gene. The constancy of the MIC values in time when strains were incubated in drug-free medium strongly supports the assumption that the occurrence of spontaneous mutations is uncommon.

Diminished sensitivity of the target is probably the main resistance mechanism to azoles in *C. parapsilosis*. Likewise, phenotypically stable resistance to azole antifungals in *C. albicans* can result from mutations in genes involved in the ergosterol synthesis pathway (including the target enzyme 14-α-demethylase). Regarding *C. parapsilosis*, the *ERG* sequences of the biosynthetic pathway of ergosterol need to be determined in resistant strains and compared with susceptible strains to clarify whether mutations are associated with azole resistance, as well as to assess changes in membrane

lipid content. Studies involving a large number of isolates of *C. parapsilosis*, aiming to characterize the cellular and molecular factors conferring resistance are being pursued.

In summary, we described the rapid and stable development of azole resistance in *C. parapsilosis* after growth in the presence of azole antifungals, fluconazole being the fastest inducer while posaconazole was the slowest.

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BRIEF REPORT

Evaluating the resistance to posaconazole by E-test and CLSI broth microdilution methodologies of *Candida* spp. and pathogenic moulds

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Abstract E-test methodology was compared with Clinical and Laboratory Standards Institute (CLSI) broth microdilution, particularly concerning the detection of resistance to posaconazole among clinical fungal isolates. The susceptibility of a large set of fungal strains (n=300) was evaluated following 24 and 48 h in two different culture media (RPMI 1640 and Sabouraud agar). Fungal strains were highly susceptible to posaconazole; however, few less susceptible strains were found, mostly regarding *Candida albicans*, *Candida glabrata*, *Acremonium* sp., *Cladosporium* sp. and *Scedosporium apiospermum*. Broth microdilution and E-test methods provided similar results for posaconazole-susceptible strains, while the less susceptible fungal strains (10.3% of the strains showed MIC ≥2 μg/mL) resulted in higher discrepancies between the two methodologies, particularly concerning *Candida* spp. E-test susceptibility values were critically affected by the pH of the culture media. Sabouraud medium provided similar susceptibility results for moulds to those for RPMI, soon after 24 h. Posaconazole resistance was rare in this study, but routine susceptibility methods, such as the E-test, should be able to detect fungal strains with reduced susceptibility. E-test methodology still needs improvements to recognise accurately strains less susceptible to posaconazole.

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Introduction

The E-test is an averagely priced susceptibility method widely used in clinical laboratories that is easy to perform and does not require specialised equipment. However, recent conflictive results regarding E-test antifungal susceptibility encouraged additional studies concerning the detection of antifungal resistance [1]. The susceptibility profile of several clinical strains of *Candida* spp. and moulds to posaconazole (POS) was determined by broth microdilution (BMD) and E-test methods. Different agar media (RPMI 1640 and Sabouraud) and pH conditions were tested.

Materials and methods

Three hundred strains were studied, corresponding to 131 *Candida* isolates (*C. albicans*, n=75; *C. parapsilosis*, n=13; *C. tropicalis*, n=12; *C. glabrata*, n=14; *C. guilliermondii*, n=7; *C. krusei*, n=7; and *C. lusitaniae*, n=3), 145 *Aspergillus* isolates (*A. fumigatus*, n=49; *A. flavus*, n=25; *A. niger*, n=25; *A. terreus*, n=24; *A. nidulans*, n=14; and *A. glaucus*, n=8), and 24 additional mould isolates (*Mucor* sp. n=2; *Rhizopus* sp., n=2; *Acremonium* sp., n=3; *Scedosporium apiospermum*, n=3; *Cladosporium* sp., n=3; *Tricho-*

Table 1 Agreement (%) between the Clinical and Laboratory Standards Institute (CLSI) broth microdilution and E-test® susceptibility methods performed with home-made RPMI 1640 agar culture medium (pH 7.0) and Sabouraud agar (pH 6.0), after incubation for 24 and 48 h

Strains (*n*)	RPMI 1640 (24h)	RPMI 1640 (48h)	Sabouraud (24h)	Sabouraud (48 h)
Susceptible strains [a]				
Candida spp. (108)	93%	88%	83%	83%
Aspergillus spp. (145)	89%	93%	92%	83%
Other moulds [b] (16)	75%	80%	75%	54%
Less susceptible strains [a]				
Candida spp. [c] (23)	27%	10%	25%	25%
Moulds [d] (8)	75%	75%	86%	100%

[a] Broth microdilution minimal inhibitory concentrations (MICs) were determined for zygomycetes at 24 h, for *Scedosporium apiospermum* at 72 h and for *Candida* spp. and *Aspergillus* spp. at 48 h (CLSI protocols M27-A3 and M38-A2)

[b] Including *Mucor* sp. (*n*=2), *Rhizopus* sp. (*n*=2), *S. apiospermum* (*n*=1), *Trichoderma* sp. (*n*=3), *Alternaria* sp. (*n*=2) and *Trichophyton* sp. (*n*=6) strains

[c] Strains with MIC≥2 μg/mL to posaconazole determined according to the CLSI broth microdilution method, including *C. albicans* (*n*=12), *C. parapsilosis* (*n*=1), *C. tropicalis* (*n*=3) and *C. glabrata* (*n*=7) strains

[d] Strains with MIC≥2 μg/mL to posaconazole determined according to the CLSI broth microdilution method, including *Acremonium* sp. (*n*=3), *Cladosporium* sp. (*n*=3) and *S. apiospermum* (*n*=2) strains

derma sp., *n*=3; *Alternaria* sp., *n*=2; and *Trichophyton* sp,. *n*=6). All strains were clinical isolates (*Candida* spp. from blood and genital tract, and moulds from lower respiratory tract and surgical wounds) obtained from the Laboratory of Pathology, Hospital S. João, Porto, Portugal. The single exception was *A. glaucus*, which was isolated from hospital air samples. Yeasts were characterised by Vitek 2 (BioMérieux, Paris, France) and moulds by macro- and micromorphology under light microscopy. Quality control strains *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were included each time as controls; the minimal inhibitory concentration (MIC) of POS was within the recommended values for both strains [2, 3]. POS powder and E-test® strips were kindly provided by Schering-Plough Farma (Cacém, Portugal). The determination of BMD MIC was performed according to the M27-A3 and M38-A2 protocols standardised by the Clinical and Laboratory Standards Institute (CLSI) for yeast and moulds respectively [4, 5]. E-test (AB Biodisk, Solna, Sweden) susceptibility was performed according to the manufacturer's instructions. The test strips were evaluated in two culture media: Sabouraud agar with 2 % glucose (Difco), pH of 6.0, and RPMI 1640 agar medium (Sigma) supplemented with 2% glucose, at pH values of 6.0 and 7.0. Two distinct RPMI agar formulations were compared: the first purchased from Izasa Portugal (Oeiras, Portugal) and the second prepared in our laboratory, as recommended by the E-test instructions. All tests were performed in duplicate.

For both methods, MICs were read following 24 and 48 h of incubation at 35ºC, with the exception of zygomycetes (after 24 h) and *S. apiospermum* (after 72 h). MICs obtained with both methods were considered to be in agreement whenever falling within no more than two consecutive dilutions [6, 7]. Fungal strains were

Table 2 Errors (%) from the comparison of the CLSI broth microdilution and E-test[a] methodologies, for the determination of posaconazole susceptibility

Strains (*n*)	VME (24h)	ME (24h)	VME (48h)	ME (48h)
Candida spp. [b] (131)	13% [d]	11%	10% [d]	12%
Aspergillus spp. [b] (145)	–	2%	–	4%
Other moulds [b,c] (24)	–	17%	–	29%

VME: very major errors; ME: major errors

[a] RPMI 1640 agar medium, after incubation for 24 and 48 h

[b] Broth microdilution MICs were determined for zygomycetes at 24 h, for *Scedosporium apiospermum* at 72 h and for *Candida* spp. and *Aspergillus* spp. at 48 h (CLSI protocols M27-A3 and M38-A2)

[c] Including *Mucor* sp., *Rhizopus* sp., *Acremonium* sp., *Scedosporium apiospermum*, *Cladosporium* sp., *Trichoderma* sp., *Alternaria* sp. and *Trichophyton* sp. strains

[d] Including *C. albicans*, *C. tropicalis* and *C. glabrata* strains

classified according to the recently proposed in vitro breakpoints (susceptible, MIC ≤1 μg/mL; intermediate, MIC of 2 μg/mL; and resistant, MIC ≥4 μg/mL) for yeast [8] and moulds [9]. In addition, discrepant results were considered a "very major error" whenever the strain was classified as susceptible by the E-test and resistant by the BMD (false susceptibility); a classification of resistance by E-test with a corresponding susceptible BMD pattern was considered a "major error" (false resistance) [6, 10].

Results and discussion

Posaconazole exhibited excellent in vitro activity against most yeast and moulds, in agreement with previous reports [1, 11]. *C. krusei*, *C. guilliermondii* and *C. parapsilosis*, often resistant or less susceptible to antifungals like fluconazole and caspofungin [12, 13], were high susceptible to POS. Few yeast strains were less susceptible to POS (MIC ≥2 μg/mL), particularly *C. albicans* (12 strains), *C. glabrata* (7 strains), *C. tropicalis* (3 strains) and *C. parapsilosis* (1 strain). All *Aspergillus* strains showed MIC <1 μg/mL, inverse to other less susceptible moulds, like *Acremonium* sp. (MIC >16 μg/mL), *Cladosporium* sp. (MIC >16 μg/mL), and *S. apiospermum*, *Mucor* sp. or *Rhizopus* sp. (with POS MICs ranging between 1 and 2 μg/mL).

The agreement between yeast BMD MIC results following 24 and 48 h was excellent (95%); thus, 48-h results were compared using E-test methodology. Two groups of fungal strains with distinct susceptibility classification to POS were defined according to BMD MIC values, as shown in Table 1. BMD and E-test methods provided similar results for yeast susceptible strains, while the less susceptible yeast strains provided high discrepancy between the two methodologies. In fact, the E-test was unable to detect high POS MICs in 9 strains of *C. albicans*, 5 strains of *C. glabrata* and 3 strains of *C. tropicalis*, after 48 h. Our results agreed with those of other studies mostly testing POS-susceptible yeast strains [7, 11, 12, 14]. The overall comparison of the BMD and E-test methods was less apparent than previously reported because of the higher percentage (18% versus less than 2% from previous studies) of less susceptible yeast strains to POS included in this study. The isolation of yeast isolates that are less susceptible or resistant to POS has not been frequently reported [1, 11], but the susceptibility methods used routinely, as is the case with the E-test, should be able to detect all fungal strains with reduced susceptibility. Around 10% of E-test MICs for *Candida* spp. resulted in "very major errors" (Table 2), being highly problematic in clinical laboratories employing a single methodology for susceptibility testing. Conversely, no "very major errors" were found with moulds, similar to previous reports [10]. The BMD and E-test methods provided similar results for all moulds tested (Table 1).

The E-test had been previously tested in distinct culture media [14], but RPMI 1640 agar remains the recommended medium. Sabouraud medium might provide a better nutrient support for several pathogenic fungi [15] and in this study it provided similar susceptibility results for moulds than RPMI 1640 medium, soon after 24 h; inversely, it did not improve E-test results for yeasts (Table 1). The discrepancy we observed between Sabouraud and RPMI 1640 culture media came from distinct pH conditions; in fact, E-test values were similar when Sabouraud and RPMI 1640 agar were equally buffered to a pH of 6.0 (data not shown). Te Dorsthorst et al. [16] had previously shown that pH critically affects in vitro antifungal susceptibility. No differences were found when comparing home-made and commercial RPMI agar media (data not shown). The occurrence of the phenomenon "no visible growth after 24 h", incidentally mentioned by previous studies regarding *C. albicans* [12], was more regularly observed testing yeast susceptibility with "home-made" RPMI agar (the E-test was repeated in these cases). The employment of a commercial formulation of RPMI culture media with more standardised pH values may favour E-test determination and improve inter-laboratory agreement.

C. albicans and *A. fumigatus* remain the predominant yeast and mould species causing invasive infection. However, a shift towards non-*albicans Candida*, zygomycetes and *Scedosporium* species, which usually show reduced susceptibility to antifungals, was recently described [10, 11]. Additionally, the resistance of azoles has been increasingly reported [12, 13] and susceptibility methods must be prepared and should be able to detect it. E-test methodology still needs improvements in order to better allow the recognition of less susceptible yeast strains by antifungals. The employment of Sabouraud medium for E-test susceptibility testing of moulds may present considerable advantages regarding early detection and may lead to more availability in clinical laboratories.

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ORIGINAL ARTICLE

Fungal infections after haematology unit renovation: evidence of clinical, environmental and economical impact

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Abstract

Objective and methods: The Haemato-Oncology Unit, Hospital S. Joao, suffered extensive refurbishing intervention in order to adapt for autotransplant patients. Eight new individual rooms with central HEPA filtration system were built. All patients admitted in the department during 14 months prior to and 14 months after renovation works were enrolled. A total of 403 admissions were considered and a detailed analysis of all patients with fungal infections, air quality and antifungal consumption were evaluated in order to study clinical, environmental and economical impact after unit renovation. *Results:* Patients with acute myeloid leukaemia submitted to induction treatment were the most susceptible to acquisition of fungal infections. Fungal infections were reduced after installation of HEPA filters in individual rooms, particularly proven and probable fungal infections. No patients were diagnosed with proven or probable mould infection in the period after the unit renovation and no deaths were registered among patients with the diagnosis of possible fungal infection. Considering the group of patients diagnosed with fungal infection, the average of hospitalization was reduced 3 d in the latter period. The new high-protected rooms showed a reduction of 50% and 95% of airborne fungi, respectively in the first week and after the second week. The consumption of voriconazole and caspofungin was reduced, respectively, 66% and 59% and the final cost with antifungal therapy was reduced by 17.4%. *Conclusions:* Autotransplant patients may be under higher risk of infection, however, the installation of high-protective measures may efficiently prevent fungal infections in these patients. Renovation of haematology unit resulted in major clinical, environmental and economical improvements. The definition of reference values for airborne agents in hospital facilities remains urgent.

Key words acute leukaemia; antifungal therapy; fungal infection; high efficiency particulate air filters; indoor air quality

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Patients with haematological diseases are usually under intense suppressive therapies, which affect their immune status and frequently cause neutropenia and increase the risk of infection (1–3). Nosocomial fungal infections are often associated to considerable mortality and longer stay in intensive care units, being responsible for extremely high healthcare costs (1, 4, 5). The implementation of preventive infection control measures, particularly the high efficiency particulate air (HEPA) filters, may reduce the incidence of fungal infections like invasive aspergillosis (6–9). However, this reduction was not consistently observed in all clinical units (10–12). A systematic review has recently reported no significant advantages of HEPA filters in reduction of the mortality rate among haematological patients (13).

The Department of Clinical Haematology of Hospital S. Joao, Porto, initiated bone marrow autotransplant interventions in 1996, particularly in the Neutropenic Patients Unit. In order to increase the department's transplant capacity, the Haemato-Oncology Unit had

undergone restructuring works to build eight isolation rooms with HEPA filters and positive pressure central system. Are these protective measures enough to prevent fungal infections in patients admitted for autotransplant? Were clinical and environmental variables improved and patients admitted in a safer unit? The main objective of the current study considered the detailed analysis of the clinical data of all patients with fungal infections admitted at Haemato-Oncology Unit, before and after renovation works. We intent to verify if a more protected environment may be correlated with clinical improvements regarding fungal infection in risk patients admitted in haematological units. The incidence of yeast and mould infections in haematological patients, mortality rate and related risk factors was evaluated. The assessment of indoor air quality and antifungal consumption were also considered, before and after unit renovation.

Methods

Unit conditions and study population

The renovation works and installation of central HEPA filters at the Haemato-Oncology Unit started at November 2004, being the new rooms available for admission of patients in June 2005. Renovation works occurred into the Haemato-Oncology Unit separated from the main hall of the unit by a barrier (door). No additional measures were considered. The installed air filtration HEPA system was based on a polyester pre-filter G4 (initial efficiency of 70%) replaced every 15 d, a polyester fine filter F8 (24×24 inches; flow of 4500 $m^3\ h^{-1}$; Luwa GmbH, Frankfurt, Germany) replaced every 6 months and the HEPA filter H13 of polyester and fiberglass (glued type with a flow of 4200 $m^3\ h^{-1}$; Camfil Farr, Trosa, Sweden) replaced once a year. HEPA air filtration system was turned on continuously 24 h per day, being turn off only for filter replacement, and it was adjusted to 15–20 air exchanges per hour. Additional protective conditions were added to the new rooms like the existence of an anteroom with HEPA filters installed along the entrance to each room (during door opening prevents air flow into patient room, also preventing the access of non-authorized people), water filtration and the systematic use of protective sterile clothes (the unit staff or visitors must wear sterile gown, shoe covers, head cap and surgical mask). The department availability comprised 12 beds before renovation works (one room each for six men and six women, respectively, both without air filters or other protective measure) and 14 beds after the unit renovation (two rooms with three beds each, both without air filters or other protective measure, plus the eight new individual rooms equipped with HEPA filters and positive air flow). Additional protective measures like extensive hand disinfection were part of the staff routines. Patients and unit staffs were continuously alerted to a higher risk of infection during renovation works and stressed to keep the wards maximally isolated from the exterior air. During that period, visitors and unit staff must nevertheless wear surgical facial mask into the rooms with no protective measures, the doors and windows were always kept closed, the patients only leaving the room for clinical exams and to use toilet facilities. In such cases, patients must wear N95 NIOSH facial mask (Kimberly-Clark SL, Madrid, Spain), as recommended (14). All patients admitted in the department from April 2004 to July 2006 were monitored, corresponding to the period before and during the works (14 months) and the period of 14 months immediately after the renovation works. The Haemato-Oncology Unit admitted patients with all types of acute leukaemia and the most severe cases of lymphoma/myeloma malignancies in the northern region of Portugal. Data regarding haematological disease, admission diagnosis, length of stay at hospital, duration of neutropenia, acquired infections, therapeutic protocols and origin of infection were registered.

Definition of hospital-acquired infection

The definition of invasive fungal infection was established in three levels, proven, probable and possible, according to previous consensual criteria (15). Hospital-acquired yeast infections or nosocomial infections were defined following the isolation and diagnosis of infections after the initial 48 h after admission in the haematology unit, according to Centers for Disease Control and Prevention (CDC) (16) and other reports (17). Prolonged fever corresponded to the persistence of fever for more than 4 d and refractory to broad-spectrum antibacterial treatment, while prolonged neutropenia corresponded to neutropenia longer than 10 d (15). The incidence of fungal infections was determined considering the number of admitted patients diagnosed with fungal infection against the total number of admissions.

Antifungal consumption

The antifungal agents requested by the Haemato-Oncology Unit during the 28 months surveillance period were quantified and the consumption associated to amphotericin B, both deoxycholate and liposomal formulation, fluconazole, voriconazole and/or caspofungin was evaluated. The same price was considered for each antifungal formulation in the periods before and after renovation works, in order to avoid false comparisons due to antifungals price adjustments along the 28 months.

Environmental surveillance

The conventional volumetric air sampling method using the Andersen one-stage sieve impactor was used for air surveillance. This standard method is recommended to collect and quantify airborne fungal species. Eighteen impactor air samples with six different impact volumes of air (14, 28, 56, 84, 140 and 280 L) were collected in each room, at a flow of 28 L/min, as recommended (18). Larger air volumes were not collected due to dehydration of agar culture medium for prolonged air exposure. Impactor air samples were cultured using the DG18 medium (19, 20) and incubated at 25°C and 37°C, during 10 d. Results were expressed as number of colony-forming units per cubic meter (CFU/m^3) of analyzed air. Regression through the origin was applied to the data and the concentration of CFU in the analyzed air determined from the slope of the regression line. For each room, the total number of colonies was plotted against the volume of analyzed air. Identification of fungal colonies was based upon macro and microscopic morphological aspects, accordingly the standard mycological methods (21). Sample collection was conducted in phases of four consecutive weeks each (sampling phases performed every 3 months), before and after the renovation works.

Statistical analysis

SPSS 13.0 (SPSS Inc., Chicago, IL, USA) application was used for data elaboration and analysis. Student's *t*-test for paired and two independent samples was used during statistical analysis. The comparison of infection incidence in the periods before and after renovation works was performed determining *z*-values, the population considered at normal distribution. Alpha was set to 0.05 and all reported *P*-values were two tailed.

Results

Haematological malignancies and fungal infections

A total of 403 admissions (198 before and 205 after the renovation works), corresponding to 221 patients (119 women and 102 men), were made to the Haemato-Oncology Unit during the 28 months surveillance period. Patients' age ranged between 15 and 86 years old (average of 54 years old). The haematological patients admitted at the unit corresponded to 75 patients with acute myeloid leukaemia (33.9%), 25 with acute lymphoblastic leukaemia (11.3%), 4 with blast crisis of chronic myeloid leukaemia (1.8%), 4 with chronic lymphoblastic leukaemia (1.8%), 5 with myelodysplastic syndrome (2.3%), 1 with plasma cell leukaemia (0.5%), 11 with Hodgkin lymphoma (5.0%), 51 with non-Hodgkin lymphoma (23.1%), 35 with multiple myeloma (15.8%) and 10 with aplastic anaemia (4.5%). The admissions of both periods are more detailed in Table 1.

Table 1 Admissions, duration of neutropenia, unfavourable outcome and fungal infections in haematological patients, before and after unit renovation

	Before renovation	After renovation
Admissions	198	205
Non-neutropenia	51	43
Short neutropenia	68	74
Long neutropenia	79	88
Deaths	16	8
Fungal infections (FI)	13	10
Yeasts[1]	3	0
Moulds[1]	3	0
Possible FI	7	10

[1]Proven and probable infections.

The incidence of fungal infections in haematological patients admitted at unit was 6.6% and 4.9% ($P = 0.001$), respectively, in the periods before and after the renovation works, corresponding to 0.33 patients per 100 d before works and 0.26 patients per 100 d after installation of protective measures (Table 1). These fungal infections also corresponded to 0.64 infections per 100 d of neutropenia before renovation and 0.49 infections per 100 d of neutropenia in the latter period. Mould infections, proven and probable, were significantly reduced after the renovation works (incidence of 1.5% and 0%, respectively, before and after renovation; $P < 0.001$), as well as proven yeast infections (incidence of 1.5% and 0%, respectively, before and after renovation; $P < 0.001$). The details of the patients diagnosed with proven and probable fungal infections are described in Table 2. Patients 1 and 6 stayed at haematology unit during the construction period, while the other four patients were admitted and left the unit before this period. Pre-construction period showed more fungal infections compared to construction period (incidences of 9.5% vs. 3.7%) probably due to the decrease of admitted patients for treatments causing longer expected neutropenia. After the unit renovation, there were no proven or probable fungal infections, being diagnosed 10 cases of possible fungal infection (in the previous period of 14 months, seven cases were diagnosed, Table 1). Of the previous 17 cases, three were PCR positive for *Aspergillus fumigatus*. However, this test is not still accepted as microbiological criteria. Nevertheless, it was especially relevant to the fact that there were no deaths in patients with the diagnosis of possible fungal infection in the latter period against three patients with poor outcome in the first 14 months (Table 1). More severely ill patients

Table 2 Data from haematological patients diagnosed with proven and probable fungal infections (all patients presented fever for more than 4 d, submitted to broad-spectrum antibiotics)

	Neutropenia (days)	Haematological disease	Diagnosis of fungal infection	Fungi detected	Level[1]	Outcome
Patient 1	20	Acute myeloid leukaemia	CT scan suggesting fungal infection, negative antigen test, positive biopsy to *A. fumigatus*	*Aspergillus fumigatus*	Proven, deep tissue	Alive
Patient 2	27	Acute myeloid leukaemia	Positive cultures from blood and bronchial secretions to *C. glabrata*	*Candida glabrata*	Proven, fungemia	Died
Patient 3	22	Acute myeloid leukaemia	Positive blood culture	*Saccharomyces cerevisiae*	Proven, fungemia	Alive
Patient 4	20	Acute myeloid leukaemia	Positive cultures from catheter and blood	*Candida parapsilosis*	Proven, fungemia	Alive
Patient 5	27	Acute myeloid leukaemia	Two CT scans suggesting fungal infection, three positive cultures to *A. fumigatus*, two positive antigen tests, one positive PCR test	*Aspergillus fumigatus*	Probable	Died
Patient 6	6	Non-Hodgkin lymphoma	Symptoms of lower respiratory tract infection and pleural infusion, two positive cultures from bronchial secretions to *A. fumigatus*	*Aspergillus fumigatus*	Probable	Alive

CT, Computerized tomography.
[1]According to Ascioglu *et al.* (15).

were admitted during the latter period in the new protected wards compared to the wards without HEPA filters. The 10 cases of possible fungal infection detected after the renovation works corresponded to 7 patients admitted in the new rooms with HEPA filters and 3 patients admitted in the other rooms without air filtration. Haematological patients with expected prolonged neutropenia (more than 10 d) or submitted to autotransplant were preferentially admitted in the new rooms. All patients diagnosed with fungal infection stayed at least 3 wk into the haematology unit and were frequently neutropenic for longer periods (the average was 23 d in both studied phases), being simultaneously under administration of large spectrum antibiotics due to the systematic isolation of other microbial agents from clinical samples and/or central venous catheters.

Patients with acute myeloid leukaemia submitted to the induction treatment were more susceptible to acquisition of fungal infections, both yeast and mould infections, compared with patients with other haematological malignancies and/or submitted to other treatments. A single case of probable mould infection was detected in a patient diagnosed with non-Hodgkin lymphoma (patient 6 shown in Table 2), being also found during the entire study a single case of possible fungal infection in each group of haematological patients admitted with acute lymphoblastic leukaemia, myelodysplastic syndrome, aplastic anaemia and non-Hodgkin lymphoma. Thirty-eight autotransplants were successfully performed after the renovation works; these patients were never diagnosed with a fungal infection. *A. fumigatus* was the most frequent and detected mould during the 28 months surveillance period, being registered a single case of possible infection by Mucorales in a patient admitted before the unit renovation. The three described yeast infections were all caused by different organisms. Two cases were classified as nosocomial yeast infections (patients 2 and 3; Table 2).

Economical impact

Considering the group of patients diagnosed with all fungal infections, the average of hospitalization days was reduced 3.4 d in the latter period. Patients with a proven or probable fungal infection stayed from 21 to 75 d in haematology unit (average of 41 hospitalization days). Although there was also a reduction in the number of patients diagnosed with a fungal infection, the administration of some antifungal agents did not follow this tendency. The consumption of voriconazole and caspofungin in the Haemato-Oncology Unit was reduced 66% and 59%, respectively, in the period after the renovation works, while increased the use of deoxycholate amphotericin B, liposomal amphotericin B and fluconazole (Table 3). The final cost with antifungal therapy was reduced by 17.4% (around €71 000) during the second arm of the study. The usual choice for empiric antifungal therapy of moulds was an amphotericin B formulation,

Table 3 Consumption of antifungal agents before and after renovation of haematology unit (number of packs)

Antifungal and formulation	Before	After	Difference (%)
Deoxycholate amphotericin B (50 mg)	580	921	+59
Liposomal amphotericin B (50 mg)	759	990	+30
Fluconazole (200 mg), capsules	691	3342	+484
Fluconazole (2 mg mL^{-1}), intravenous formulation (200 mL)	124	180	+45
Voriconazole (200 mg), capsules	454	330	−28
Voriconazole (200 mg), intravenous formulation	138	7	−95
Caspofungin, intravenous formulation	382	158	−59

being the second line therapy mainly an association of drugs using voriconazole and/or caspofungin. For yeast infections, the first choice was regularly fluconazole and the alternative caspofungin or liposomal amphotericin B. Liposomal amphotericin B was administered at 3 mg kg day^{-1}. The daily defined dose of the other antifungals administered to patients was similar in both periods and established according to standard therapeutic protocols.

Environmental surveillance

Higher values of *A. fumigatus*, *Aspergillus flavus* and *Aspergillus niger* were detected in the cultures incubated at 37°C, while the other fungal species were mostly detected at 25°C. The air surveillance of the clinical unit disclosed values of total fungi ranging from 22 to 278 CFU/m^3 (average of 131 CFU/m^3) in the rooms without air filtration system along the 28 months surveillance period. In the same wards, *A. fumigatus* ranged between 0.7 and 19.3 CFU/m^3 (average of 8.5 CFU/m^3). The haematology main hall was not monitored during the entire study, including the construction period. Airborne quality was assessed exclusively in patients' wards throughout the study, being all rooms considered. Staff and patients were continuously alerted to keep wards maximally isolated from outdoor air. No significant differences were found during the renovation works considering airborne fungal levels into such wards ($P > 0.05$). However, the levels of *A. fumigatus* increased from 8.5 to 12 CFU/m^3, during the construction period. The new rooms with HEPA filters showed a gradual improvement of the air quality since the first week (Fig. 1). The total airborne fungi decreased from 70 CFU/m^3 (reduction of 50%) in the first week to less than 7 CFU/m^3 (reduction of 95%) in the following weeks. *Aspergillus fumigatus* yielded 1.4 CFU/m^3 in the first week but it was not detected thereafter. Similar corresponding airborne values were detected in the entrance hall (also with HEPA filters) that conducted to the patient protected rooms (data not shown).

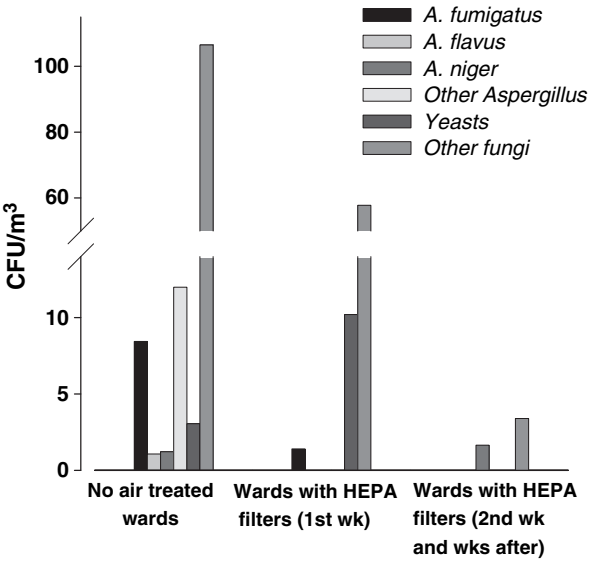

Figure 1 Colony-forming units per cubic meter (CFU/m^3) of fungi detected during air quality surveillance in haematology unit after renovation works. Other fungi included mostly *Penicillium* sp. and rare isolates of *Mucor* sp., *Alternaria* sp. and *Scedosporium* sp. Values of *Aspergillus fumigatus*, *Aspergillus flavus* and *Aspergillus niger* were detected in the cultures incubated at 37°C, while the other fungal species were mostly detected at 25°C.

Discussion

Construction and renovation works are a well-known risk factor for mould infections (6, 22, 23). *Aspergillus* infections resulting from failure in air filtration systems have been reported (24, 25). Other studies have reported a reduction of *Aspergillus* infections after the installation of HEPA filters and/or by the existence of physical barriers that limit the access of airborne conidia to the clinical units (6–9). The installation of HEPA filters in the individual rooms did not completely prevent fungal infections in the haematological patients admitted in our clinical unit, although its incidence was reduced by 25%. HEPA filters had shown in other studies a reduction of airborne fungi to less than 10 CFU/m^3 (26) and a reduction of mould infections in haematological patients (7). After unit renovation, the new rooms with HEPA filters showed a large improvement of air quality compared with the non-treated rooms, as well as a clinical positive impact – no proven or probable fungal infections were registered in the latter period and no deaths associated to possible fungal infections were reported, even considering the 38 autotransplants performed in the unit during this period. During the construction period, a small increase of airborne *A. fumigatus* was found

but there was no significant clinical impact compared with the previous months, conversely to what had been shown in other locations (6, 22, 23). Even after the installation of HEPA filters, it was possible to find moulds into protected wards, particularly *Penicillium* sp., but these species are not commonly pathogenic, with the exception of *Penicillium marneffei*. *Aspergillus* species were rarely found, particularly *A. fumigatus*. Sherertz *et al*. had reported no *A. fumigatus* infections in clinical environments with less than 0.1 CFU/m^3 (27). However, reference airborne fungal values are still not defined, as well as it is not yet established the sampling air frequency that should be performed in clinical units. Confirming previous reports (4), a higher number of fungal infections were reported among patients with acute myeloid leukaemia. These patients were commonly associated to prolonged neutropenia and longer hospital stay. Incidence of invasive aspergillosis had been previously associated with an increasing number of hospital admissions per year (5, 28). Possible confounders may be detected in this study, namely regarding patients' age, patient's genetic predisposition for infection, severity of haematological malignancy and mortality rate of a unit external group. All patients staying more than 24 h in Haemato-Oncology Unit were included in this study (criteria for admission were similar in both periods) and previous mentioned factors occasionally may not be equally distributed in both compared groups (before vs. after unit renovation). However, the presented main results and values were similar to previous reports and focus different aspects, all indicative of clinical and environmental improvements after installation of protective measures.

The incidence of yeast infections was lower after renovation works, both classified as nosocomial and community-acquired infections. It is relevant that the new eight rooms were individual rooms therefore reducing inter-patient, medical staff–patient and visitor–patient contacts. Yeasts are usually present in the human internal milieu, being some nosocomial infections a clear consequence of host neutropenia and further invasion by the endogenous fungal agent.

The economical benefits of the haematology unit renovation were shown by the reduction of the hospitalization days in a particular group of patients – hospitalization costs are commonly described the most important healthcare costs (5, 29–32) – and the reduction of the expenses with antifungal agents into the unit. The larger consumption of both amphotericin B formulations and fluconazole in the latter period was most probably related to the antifungal prophylaxis administered to patients with acute myeloid or lymphoblastic leukaemia under longer neutropenia (more admissions in this period, as shown in Table 1). The administration of antifungal prophylaxis was a clinician decision and it was mostly employed in acute leukaemic patients under longer neutropenia periods, being sometimes also related to clinical history of invasive aspergillosis. Unit policy regarding prophylaxis or treatment of fungal infections was not modified during the studied period, fluconazole for yeast and amphotericin B for mould infections.

In accordance with previous studies (4, 27, 33), patients staying in hospital under prolonged neutropenia (more than 10 d) and with longer stay than 3–4 wk are more susceptible to develop fungal infections, usually associated to the isolation of other microbial agents and administration of a large spectrum of antibiotics. Patients submitted to autotransplant may be under higher risk of infection in haematological units, nevertheless, the installation of high-protective measures may efficiently prevent fungal infections in these patients, as we had shown in this study. It is urgent to understand the presence and transmission of fungal agents in protected clinical environments in order to improve health and well-being of patients submitted to immunosuppressive treatments and staying longer at hospitals.

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Conflict of interest

The authors declare no conflict of interest.

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O118 Effective reversion of fluconazole resistance by ibuprofen in an animal model

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Objectives: Ibuprofen was found to be an efficient reverter of in vitro fluconazole resistance due to overexpression of efflux pumps1,2; however its in vivo effect is still unproven.The aim of our study was to evaluate in an animal model the effect of ibuprofen associated to fluconazole in the treatment of an invasive infection by a resistant *C. albicans* isolate.

Methods: A *C. albicans* resistant (R) strain to fluconazole was obtained by subculturing with serial concentrations of fluconazole a susceptible strain (S) during 30 days. Minimal inhibitory concentrations (MIC) to fluconazole was determined in the presence of 100 μg/mL of ibuprofen (IBU), an efflux pump blocker1,2.

Comparative transcriptome analysis between the S and the induced resistant strain (R) incubated with and without ibuprofen (RI) was performed using *C. albicans* DNA microarrays from Agilent Technologies.

The in vivo study was carried out according to the murine candidiasis model. Female BALB/c mice were infected with 5 × 05 cells in 0.1 mL of sterile saline via the lateral tail vein with the S strain (three groups) or the R strain (three groups). Antifungal therapy was administered intraperitoneally with FLC or IBU or FLC + IBU on both groups 3 hours after microbial challenge and repeated once a day for a total of four days. The kidney fungal burden was determined.

Results: Ibuprofen decreased azole MIC values, the R phenotype changing to *S. Microarray* analysis identified 836 and 1517 with differential expression in R and RI strains, respectively. The R strain showed overexpression of CDR11, ERG251, CDR4 and the transcription factor UPC2. In the RI and in the S strains those genes were down regulated.

FLC showed to be effective only in the treatment of the infection by the S strain, reducing dramatically the fungal burden. Interestingly, in mice infected with the R strain but treated with FLC + IBU, a significant decrease in the fungal burden was observed. In the absence of FLC, IBU did not display antifungal activity per se.

Conclusions: The in vivo synergic effect between fluconazole and ibuprofen demonstrated herein may represent a hopeful future approach for a better management of antifungal resistance conferred by efflux pump overexpression.

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P280

Adrenaline enhances yeast cell growth and ATP production through a common target to mammalian cells

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Vasoactive amines like adrenaline are life saving medications. To which extent concomitant therapy may influence clinical effectiveness of antifungal drugs still remains unclear. In humans, the adrenergic receptors, a class of G protein-coupled receptors (GPCRs), represent catecholamine targets. Several studies have described the existence of G-protein coupled receptors in yeasts, showing an identical GTP binding site to human GPCRs. Considering the many homologies already described between yeasts and human cells we raised the hypothesis that in *Candida albicans* adrenaline could act through similar adrenergic receptors and influence ATP production by the mitochondrial respiratory chain and consequently increase efflux activity of antifungal drugs like azoles. Our aim was to clarify such effect and to elucidate about the membrane receptor involved.

Material and Methods: Strains: Six fluconazole (FLC) susceptible *Candida albicans* clinical strains (from blood cultures), wild type strain SC5314, *C. albicans* strains with selective deletion of efflux pumps genes cdr1Δ/Δ (DSY448), cdr2Δ/Δ (DSY653) and cdr1Δ/Δ cdr2Δ/Δ (DSY654), C. albicans strains gpr1 Δ/Δ and gpa2 Δ/Δ, Δ ras1-2/ Δ ras1-3 and ste2 Δ/Δ, ste3 Δ/Δ and ste4 Δ/Δ and respective wild types were used in this study.

Effect of adrenaline upon Rhodamine 6G (Rh6G) extrusion: the quantification of Rh6G efflux was performed in all strains after incubation with increasing concentrations of adrenaline (0, 5, 27, 54, 108, 136, 273 and 545 μM) for 90 min at 35 °C. The cells were stained with 5 μM of Rh-6G and analyzed in a flow cytometer (FACSCalibur BD, Bioscience, Sydney). The percentage of Rh6G intracellular accumulation was calculated and comparedamong clinical, deleted and wild type strains.

Effect of adrenaline upon oxygen consumption: the O_2 consumption was continuously monitored during 90 min with a Clark type electrode YSI model 5775 (YSI Incorporated, Yellow Springs, Ohio, USA) in the presence of 16 μg ml^{-1} fluconazole (FLC) and with 0 and 54 μM of adrenaline and the oxygen uptake rate (OUR) was calculated.

Effect of adrenaline upon yeast growth: yeast suspensions in YEPD were exposed to the following growth conditions: no treatment (control), treatment with FLC 1μg ml^{-1} (sub-MIC concentration), treatment with 0.054 and 0.108 mM of adrenaline, treatment with FLC 1 μg ml^{-1} plus 0.054 of adrenaline or with 0.108 mM of adrenaline, at 35 °C, 150 rpm. O.D.600 was registered during a 16-h period.

Results: A dose-dependent decrease of intracellular accumulation of Rh6G was observed in clinical strains treated with adrenaline. Rh-6G staining was higher in DSY 653 than in DSY 448. No decrease in staining was observed with DSY 654 strain. Clinical and SC5314 *Candida* strains treated with FLC plus adrenaline showed a higher OUR (0.99 $gl^{-1}h^{-1}$) than with only FLC. Cell growth increased in the presence of adrenaline. In gpa2 Δ/Δ and ste3 Δ/Δ strains a dose dependent increase in the percentage of Rh6G intracellular accumulation was observed; the respective wild type strains showed opposite findings.

Conclusions: Adrenaline enhances ATP production and cell growth through a Ste3 and Gpa membrane receptor.

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Easy quantification of yeast chitin cell wall content by flow cytometry

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Flow cytometry represents an efficient and fast approach for the analysis of cell architecture and functional phenotypes, with considerable advantages over conventional methods. Taking advantage of the specific chitin dye calcofluor white (CFW) that has already been used in epifluorescence microscopy we developed a flow cytometric assay to quantitatively measure cell wall chitin content in the yeast cell wall.

Twenty two *Candida* spp and 4 *Cryptococcus neoformans* clinical isolates with distinct susceptibility profile to caspofungin, were included in this study. The *C. albicans* SC5314 wild type and the Δchs3/Δchs3 strain (with low chitin content) were used as control strains for optimization of the flow cytometric protocol. Cells were grown in YPD broth medium at 35 °C, 150 rpm, until late logarithmic phase. A 10^6 yeast cells/ml suspension in distilled water was stained with 0 (autofluorescence), 2.5, 6.25, 12.5 and 25 μg ml^{-1} of CFW for 15 min at room temperature. Afterwards, yeast cells were washed twice and the blue fluorescence emitted by 50000 cells was quantified using a BD FACSCanto™ II (Becton Dickinison, Madrid, Spain) flow cytometer. The mean intensity of fluorescence emitted from stained (positive population) and the nonstained (autofluorescence or negative) yeast cell population (obtained from three independent experiments) was analyzed and processed with FACSDiva software. For each experiment a staining index (SI) was calculated as follows: (mean intensity of fluorescence of positive population - mean intensity of fluorescence of negative population)/ 2 × standard deviation of the mean intensity of fluorescence of negative population. The chitin content of 27 clinical isolates was assessed according the described protocol, after staining with 2.5 μg ml^{-1} of CFW. Next the isolates grown for 48 h in the presence of a range of caspofungin concentrations varying from 0.03 to 256 μg ml^{-1} were tested. MICs were also determined using prominent inhibition as an endpoint corresponding to 50% (MIC_{50}). The aim was to establish a relationship between fungal chitin content and the occurrence of the paradoxical growth phenomenon registered among clinical isolates in the presence of high caspofungin levels. The best CFW concentration to differentiate chitin content among the two control strains was 2.5 μg ml^{-1}. As expected, the wild type strain displayed a significantly higher staining index ($P < 0.001$) than the chs3/chs3 strain. Variable CFW staining index values were found among strains belonging to the same species, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. neoformans* had less intra-species variation. Although no relationship between chitin content and the caspofungin susceptibility pattern was found, an association between paradoxical growth in the presence of caspofungin and chitin content was established.

This novel flow cytometry protocol is a simple and reliable assay to accurately quantify cell wall chitin in yeast cells. The accuracy of this novel methodology can predict the occurrence of the paradoxical effect, representing a valuable tool for detection of antifungal compensatory mechanisms in the presence of high echinocandin concentrations.

P835 Fungaemia by *Candida parapsilosis*: *in vivo* induction of azole resistance due to prolonged therapeutic exposure

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Objectives: A patient with *C. parapsilosis* fungaemia was submitted to fluconazole during eleven days, after which he died. Several *C. parapsilosis* isolates had been collected before, during and after initiation of fluconazole therapeutics. A microbiological characterization of the first and the last isolate was performed.
Methods: *In vitro* antifungal susceptibility testing to four azoles (fluconazole, itraconazole, voriconazole and posaconazole) was performed according to CLSI protocols. Molecular typing of the *C. parapsilosis* isolates was carried out by RAPD. The stability of resistant phenotype (acquired *in vivo*) was assessed *in vitro* through repeated incubation of the isolate without antifungal. The role of active efflux pumps in drug resistance was evaluated using flow cytometric analysis with rhodamine 6G (Rh-6G), a specific substrate for efflux pumps.
Results: The initial isolate was susceptible to all azoles, while the last recovered isolate was resistant to all four tested azoles, displaying high MIC values. Molecular typing of isolates demonstrated that the last isolate and pre-treatment isolate were isogenic. Therefore, we report, the *in vivo C. parapsilosis* acquisition of antifungal resistance, which maintained stable after incubation without antifungal. No difference in Rh-6G staining was found between the susceptible and the resistant isolate, suggesting that *in vivo* azole resistance did not involve increase in efflux pumps activity.
Conclusion: We report a clinical case of fungaemia by *C. parapsilosis* which acquired resistance to azoles after exposure to fluconazole. The resistance was characterized by an increase of fluconazole, itraconazole, voriconazole and posaconazole MIC values, which kept stable. Interestingly, azole resistance due to overexpression of efflux pumps was not confirmed. Continued fluconazole pressure was associated with the development of multi-resistance.