Driving microevolution towards azole resistance, independent from *PDR1*, in *Candida glabrata*

Romeu Filipe Nobre Viana

Thesis to obtain the Master of Science Degree in

Microbiology

Supervisors: Prof. Miguel Nobre Parreira Cacho Teixeira; Dr. Dalila Madeira Nascimento Mil-Homens

Examination Committee

Chairperson: Prof. Jorge Humberto Gomes Leitão

Supervisor: Prof. Miguel Nobre Parreira Cacho Teixeira

Members of the Committee: Prof. Nuno Gonçalo Pereira Mira

October 2018

Acknowledgments

First of all, I would like to thank my supervisor Professor Miguel Nobre Parreira Cacho Teixeira, for giving me the opportunity of becoming a part of this project and his team. He always shown a contagious enthusiasm and positivity throughout the work, which was fundamental to my motivation during this project. I would especially like to thank him for always showing his willingness and patience to help me solve all the issues that have arisen. His support was crucial for the success of the work developed in this master thesis and for my growth as an investigator.

Also, I would like to thank to my co-supervisor, Dr. Dalila Madeira Nascimento Mil-Homens for being always supportive and available regarding the work developed.

I would also like to express my gratitude o Professor Isabel Sá-Correia, for allowing me to develop project at the BSRG laboratory, and for allowing me to be part of its incredible team.

My acknowledgements are also going to several parts which contributed to the achievement of this thesis. Professor Hiroji Chibana and Professor Thomas Edlind for the supply of some of the mutant strains used in this project. Dr. Acácio Rodrigues and Dr. Isabel Miranda, from Hospital de São João (Porto), for providing part of the clinical isolates and also to Dr. José Melo-Cristino, from Hospital de Santa Maria (Lisboa), for the remaining isolate collection used in this Work. Professor Arsénio Fialho for his contribution to this project and Andreia Pimenta which help me in several steps of my work. This work was supported by FEDER and “Fundação para a Ciência e a Tecnologia” (FCT) (Contracts PTDC/BBB-BIO/4004/2014 and PTDC/BII-BIO/28216/2017) and Programa Operacional Regional de Lisboa 2020, contract LISBOA-01-0145-FEDER-022231. Funding was received by iBB – Institute for Bioengineering and Biosciences from FCT (UID/BIO/04565/2013) and from Programa Operacional Regional de Lisboa 2020 (Project N. 007317).

Also, I would like to thank to my entire work team, especially to Mafalda Cavalheiro, was always supportive and available during all my lab work and Pedro Pais, for always being available to help me and for his expertise in bioinformatics. Were both very important for my growth as an investigator.

Finally, I would like to express my gratitude to Mafalda Vaz, for always believing in me, for its encouragement and assistance, and for spending the time of her summer vacation being my company while writing my dissertation. And not at least, special thanks belong to my family for their constant support and trust in me.

Abstract

Key-words: Azole resistance, Beyond *PDR1*, *C. glabrata*, Microevolution

Candida glabrata is the second most common cause of invasive candidiasis, partly due to its ability to acquire azole drug resistance, mostly through the acquisition of Gain-of-Function mutations in Pdr1, the transcription factor that controls multidrug efflux pumps.

In this work, the possible existence of azole resistance mechanisms, beyond the Pdr1 network, was inspected. Four *C. glabrata* deletion mutants, devoid of Pdr1 or alternative mechanisms of resistance under study in our lab, *Δtpo3*, *Δrpn4*, *Δpdr1* and *Δmrr1*, were selected for in vitro fluconazole resistance evolution experiments. All strains became resistant to fluconazole, independently of *PDR1* GOF mutations or of increased expression of the drug efflux transporter gene *CDR1*. Additionally, the occurrence of *CgPDR1*, *CgRPN4* and *CgMRR1* mutations was investigated in a collection of *C. glabrata* clinical isolates, confirming that there are fluconazole resistant isolates without *PDR1* GOF mutations. Interestingly, a *CgRPN4* point mutation was identified as specific of azole resistant strains. Fluconazole resistance acquisition in *Δpdr1* mutants was analyzed through genome sequencing, leading to the identification of 36 mutations that may underlie this process.

This study provides interesting new clues on specific mutations that may underlie azole resistance acquisition of expected clinical relevance, beyond the Pdr1 network.

Resumo

Palavras-chave: *C. glabrata*, Microevolução, Para além do *PDR1*, Resistência a azóis

Candida glabrata é a segunda causa mais comum de candidíase invasiva, em parte devido à sua habilidade em adquirir resistência ao fluconazole, principalmente através da aquisição de mutações de ganho de função no Pdr1, fator de transcrição que controla as *multidrug efflux pumps*.

Neste projeto, a possível existência de mecanismos de resistência a azóis, para além da rede do Pdr1, foi investigada. Quatro mutantes de deleção de *Candida glabrata*, desprovidos do Pdr1 ou de mecanismos alternativos de resistência em estudo no nosso laboratório, *Δtpo3*, *Δrpn4*, *Δpdr1* e *Δmrr1*, foram selecionados para testes de evolução de resistência ao fluconazole *in vitro*. Todas as estirpes tornaram-se resistentes ao fluconazole, independentemente de mutações de ganho de função no *PDR1* ou expressão aumentada do gene *drug efflux transporter CDR1*. Adicionalmente, a ocorrência de mutações nos genes *CgPDR1*, *CgRPN4* e *CgMRR1* foi investigada numa coleção de isolados clínicos de *C. glabrata*, confirmando a existência de isolados resistentes ao fluconazole sem qualquer mutação de ganho de função no *PDR1*. Surpreendentemente, uma mutação pontual no *CgRPN4* foi identificada como sendo especifica de estirpes resistentes a azóis. A aquisição de resistência ao fluconazole nos mutantes *Δpdr1* foi analisada através da sequenciação do genoma, permitindo a identificação de 36 mutações que poderão estar por detrás deste processo.

Este estudo fornece interessantes novas pistas sobre mutações específicas que poderão estar por detrás da aquisição de resistência a azóis com uma esperada relevância clínica, para além da rede do Pdr1.

Table of Contents

List of Figures

List of Tables

Abbreviations

ABC	ATP-binding-cassette
AD	Putative transcriptional activation domain
AIDS	Acquired immunodeficiency syndrome
A. fumigatus	*Aspergillus fumigatus*
AUC	Area under the curve
AKR	Aldo-keto-reductase
BSIs	Bloodstream infections
cDNA	Complementary deoxyribonucleic acid
Ca	*Candida albicans*
Cg	*Candida glabrata*
C. neoformans	*Cryptococcus neoformans*
C. albicans	*Candida albicans*
C. glabrata	*Candida glabrata*
C. krusei	*Candida krusei*
CFU	Colony forming units
CGD	Candida genome database
CHSJ	Centro Hospitalar São João
CLSI	Clinical and Laboratory Standards Institute
CTG	Codon usage transition
DBD	DNA binding domain
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
DUF	Domain of unknown function
EDTA	Ethylenediamine tetraacetic acid
FAD	Flavin adenine dinucleotide
FCT	Fundação para a Ciência e Tecnologia
FLF	Flavodoxin-like fold domain
FSTFD	Fungus-specific transcription factor domain
GOF	Gain-of-function
GPI	Glycosylphosphatidylinositol
GTP	Guanosine triphosphate
iBB	Institute for Bioengineering and Biosciences

ICU	Intensive care units
ID	Identity
HIV	Human immunodeficiency virus
HR	Heteroresistance
HSM	Hospital of Santa Maria
Mbp	Mega base pairs
MDR	Multidrug resistance
Med3	Mediator complex subunit 3 fungal
MFS	Major facilitator superfamily
MIC	Minimal inhibitory concentration
min	Minute
NADPH	Nicotinamide adenine dinucleotide phosphate
NCCLS	National Committee for Clinical Laboratory Standards
Nº	Number
OD	Optical density
ORF	Open reading frame
PAP	Population analysis profiling
PCR	Polymerase chain reaction
PDB	Protein data bank
PDRE	Pleiotropic drug response element
PFD	Prefoldin subunit
PID	Putative inhibitory domain
PIR	Yeast PIR protein repeat
PPP4R2	Protein phosphatase 4 core regulatory subunit R2
qRT-PCR	Quantitative real-time PCR
RanBP1	Seripauperin and TIP1 family domain
RNA	Ribonucleic acid
rpm	Rotations per minute
RPMI	Roswell Park Memorial Institute medium
Sc	*Saccharomyces cerevisiae*
S. cerevisiae	*Saccharomyces cerevisiae*
SDA	Sabouraud dextrose agar
SDS	Sodium dodecyl sulfate
sec	Seconds
SGD	Saccharomyces genome database
SNP	Single-nucleotide polymorphism
SP	Signal peptide

TIP1	Seripauperin and TIP1 family domain
UV	Ultraviolet
WGD	Whole-genome duplication
WH2	Wiskott-Aldrich syndrome homology region 2
YPD	Yeast extract – Peptone – Dextrose
ZF	Zinc finger domain
3D	Three-dimensional

1. Introduction

1.1 *Candida glabrata*, a current problem

Over the past several decades, there has been a drastic increase in the incidence of nosocomial fungal infections, which are expected to continue to rise in the coming decades (Perlroth et al., 2007). This increase is attributed to: an aging population in countries with advanced medical technologies; patients with long-term stays in intensive care units; the increased use of central venous catheters and immunosuppressive therapy; increased number of cancer patients undergoing chemotherapy; increased number of patients undergoing organ or hematopoietic stem cell transplantation (Perlroth et al., 2007; Strollo et al., 2016). *Candida* species are by far the most common fungi causing invasive disease in humans (table 1) (Wisplinghoff et al., 2004; Wisplinghoff et al., 2003a; Wisplinghoff et al., 2003b). Among all *Candida* species *C. albicans* is the predominant causative agent of invasive candidiasis, although in the last 3 decades an increase in the incidence of non-albicans *Candida* species has been observed, specifically *Candida glabrata* (Pfaller and Diekema, 2007).

Table 1: Percentage of incidence of pathogens most commonly isolated from monomicrobial nosocomial bloodstream infections (BSIs) for all patients and patients in intensive care units (ICU) in US Hospitals from an analysis of 24,179 Cases from a Prospective Nationwide Surveillance Study (Wisplinghoff et al., 2004).

	Percentage of BSIs (rank)	
Pathogen	**Total (n=20,978)**	**ICU (n=10,515)**
Coagulase-negative Staphylococci	31,3 (1)	35,9 (1)
Staphylococcus aureus	20,2 (2)	16,8 (2)
Enterococci	9,4 (3)	9,8 (4)
Candida species	9 (4)	10,1 (3)
Escherichia coli	5,6 (5)	3,7 (8)
Klebsiella species	4,8 (6)	4,0 (7)
Pseudomonas aeruginosa	4,3 (7)	4,7 (5)
Enterobacter species	3,9 (8)	4,7 (6)

In the past, *Candida glabrata* was considered a relatively nonpathogenic fungus of the normal flora of healthy individuals. However, following the extensive and increased use of broad-spectrum antimycotic prophylaxis together with immunosuppressive therapy, the incidence of infections caused by *C. glabrata* has increased significantly (Fidel et al., 1999). In fact, *Candida*

glabrata has recently emerged as the second most common cause of invasive candidiasis, surpassed only by *C. albicans* (table 2), and an increasing number of reports have recently been put forward showing their important role in mucosal or bloodstream infections (Pappas et al., 2003; Pfaller and Diekema, 2007).

Table 2: Distribution of *Candida* species from cases of invasive candidiasis. Data compiled from the ARTEMIS DISK Surveillance Program, 1997 to 2003. Total number of cases Includes all specimen types and all hospitals from a total of 127 different institutions in 39 countries (Pfaller and Diekema, 2007).

Species	**% of total no. of cases**					
	1997-1998	**1999**	**2000**	**2001**	**2002**	**2003**
C. albicans	73.3	69.8	68.1	65.4	61.4	62.3
C. glabrata	11.0	9.7	9.5	11.1	10.7	12.0
C. tropicalis	4.6	5.3	7.2	7.5	7.4	7.5
C. parapsilosis	4.2	4.9	5.6	6.9	6.6	7.3
C. krusei	1.7	2.2	3.2	2.5	2.6	2.7
C. guilliermondii	0.5	0.8	0.8	0.7	1.0	0.8
C. lusitaniae	0.5	0.5	0.5	0.6	0.5	0.6
C. kefyr	0.2	0.4	0.5	0.4	0.4	0.5
C. rugosa	0.03	0.03	0.2	0.7	0.6	0.4
C. famata	0.08	0.2	0.5	0.2	0.4	0.3
C. inconspicua			0.08	0.1	0.2	0.3
C. norvegensis			0.08	0.1	0.07	0.1
C. dubliniensis			0.01	0.08	0.1	0.05
C. lipolytica			0.06	0.06	0.06	0.08
C. zeylanoides			0.03	0.08	0.02	0.04
C. pelliculosa				0.06	0.05	0.04
Candida spp.c	3.9	6.0	3.7	3.3	7.9	4.9
Total no. of cases	22,533	20,998	11,698	21,804	24,68	33,002

1.2 Characterization of *Candida glabrata*

Historically, *C. glabrata* was first described as *Cryptococcus glabratus* (Anderson, 1917). In 1950 it was identified as the source of several infections and began to be also named as *Torulopsis glabrata* (Fidel et al., 1999). The *Candida* genus was described in 1913, however, due to its inability to grow as hyphae, *C. glabrata* was not classified in this genus (Fidel et al., 1999). This yeast did not receive much attention until it began to be associated with candidaemia in

immunocompromised patients (Bolotin-Fukuhara and Fairhead, 2014; Gabaldon and Carrete, 2016). Only in 1978 was it classified as *Candida glabrata* (Fidel et al., 1999), when it was clear that the production of hyphae was not a reliable distinguishing factor for the genus *Candida* (Fidel et al., 1999). In fact, *C. glabrata* does not display several virulence factors associated to other *Candida* species, namely, hyphal growth or the secretion of proteases (Kaur et al., 2005). Despite that, *C. glabrata* is a growing concern in clinical settings, being associated to a high mortality rate in compromised, at-risk hospitalized patients (Fidel et al., 1999).

On Sabouraud Dextrose Agar (SDA), *C. glabrata* forms smooth, shining, and cream-colored colonies, which are relatively indistinguishable from those of other *Candida* species, except for their relative size, *Candida glabrata* cells (1–4μm) being quite smaller than *C. albicans* (4–6 μm) (Rodrigues et al., 2014). Genetically, its haploid genome is a distinguishing characteristic, contrary to the diploid genome of *C. albicans* and several other non-albicans *Candida* species (Fidel et al., 1999).

The emergence of *C. glabrata* as a human pathogen is thought to have occurred independently from other *Candida* species (Singh-Babak et al., 2012). The genome sequence of the *C. glabrata* isolate CBS138/ATCC2001 became available in 2004 (Dujon et al., 2004), allowing the possibility of comparing its genetic constituents with that of *C. albicans* (Jones et al., 2004) and *S. cerevisiae* (Goffeau et al., 1996). Comparative genomics showed that *C. glabrata* is phylogenetically closer to the model yeast *Saccharomyces cerevisiae* than to *C. albicans*, (figure 1), descending from the same ancestor that underwent a whole-genome duplication (WGD) event (Roy and Thompson, 2015). *C. glabrata* is one of the few pathogens from this branch of the phylogenetic tree of the Saccharomycetaceae (figure 1). Kurtzman proposed in 2003 the inclusion of *C. glabrata* in the *Nakaseomyces* clade (Kurtzman, 2003). Later two new pathogens, *Candida nivariensis* and *Candida bracarensis*, were discovered and included in the same clade (Figure 1) (Alcoba-Florez et al., 2005; Correia et al., 2006). *C. glabrata* stands out as an outlayer (Santos et al., 2011), in fact, typical *Candida* pathogens belong to the *Candida* clade, a group of species characterized by a rare particularity in their genetic code: the CUG codon encodes the amino acid serine instead of leucine (Gabaldon and Carrete, 2016; Santos et al., 2011). *Candida glabrata*, in contrast, uses the standard genetic code such as the model yeast *S. cerevisiae* (Gabaldon and Carrete, 2016). Comparison of the whole-genome sequence between *S. cerevisiae* and *C. glabrata*, showed that *C. glabrata* appears to have lost many more genes than *S. cerevisiae*, including the complete loss of some metabolic pathways and decreasing traces of duplication to a minimum (Rodrigues et al., 2014). Additionally, *C. glabrata* has lost various genes, such as some involved in galactose assimilation, phosphate, nitrogen, sulfur metabolism, and pyridoxine biosynthesis (Dujon et al., 2004). As a result, it is unable to synthesize or use some nutrients and metabolites what is thought to be compensated by the mammalian host environment (Rodrigues et al., 2014).

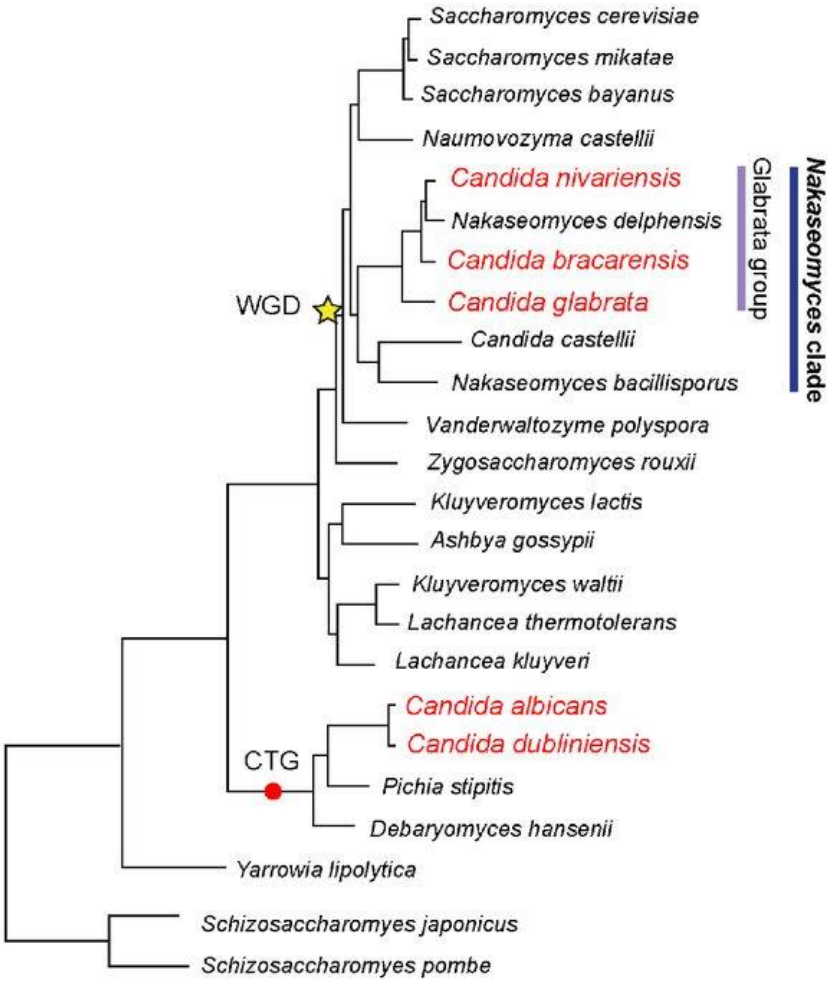


Figure 1: Phylogenetic tree representing the evolutionary relationships between sequenced Ascomycete fungal species. The human commensal/opportunistic pathogen species are listed in red type. Major evolutionary events in this phylogeny, such as the WGD (yellow star) and the codon usage transition in the *Candida* (CTG) clade, are indicated on the tree (Roy and Thompson, 2015).

1.3 Azoles as antifungal agents

For the last two decades, the azoles have been the most widely used class of antifungal drugs, due to their broad spectrum activity, low toxicity, and bioavailability (Robbins et al., 2016; Roemer and Krysan, 2014). These drugs act by blocking ergosterol biosynthesis inhibiting the cytochrome P-450-dependent (it contains a heme group located in the active site) enzyme lanosterol 14α-demethylase (Erg11), encoded by the *ERG11* gene (figure 3). This enzyme is involved in the

synthesis of ergosterol, but when bound by azole drugs it leads to an inability to build and renew ergosterol in the cellular membranes. It has been observed that in many fungal species, these drugs enter the fungal cell by facilitated diffusion (Mansfield et al., 2010) and act through an unhindered nitrogen atom in the azole ring, which binds to the iron atom of the heme group located in the active site of Erg11 (Hitchcock, 1991; White et al., 1998), leading to the inhibition of the activation of oxygen, necessary for the demethylation of lanosterol, blocking the production of ergosterol and causing accumulation of 14-α-methyl-3,6-diol, a toxic sterol produced by the Δ-5,6-desaturase which is encoded by *ERG3* (figure 4) (Lupetti et al., 2002). This inhibition leads to changes in membrane fluidity and function, affecting vital processes such as signaling, transport, exocytosis, and endocytosis (Rodrigues et al., 2014) (Ferrari et al., 2009). The azoles generally are fungistatic against *Candida* species and this allows a strong directional selection on surviving populations to evolve drug resistance (Cowen and Steinbach, 2008). Among the azoles class, fluconazole is the most commonly prescribed antifungal used to treat *Candida* infections. However, *Candida glabrata* clinical isolates are frequently resistant to fluconazole (Cowen and Steinbach, 2008).

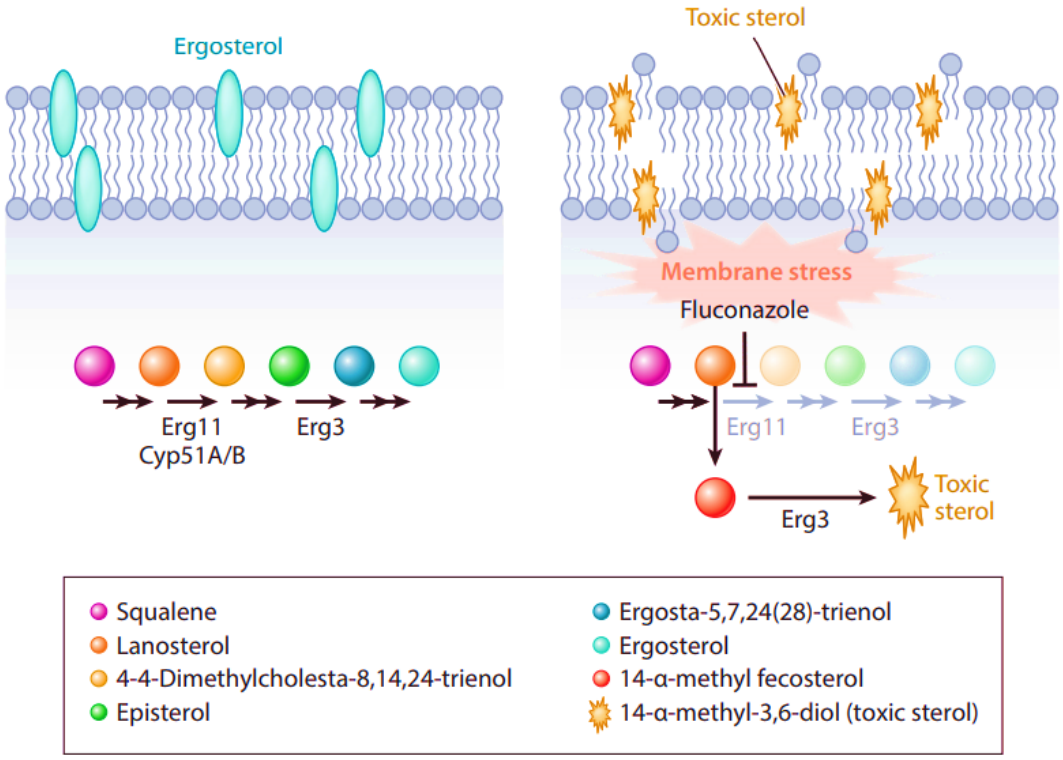


Figure 2: Mechanism of action of azoles from (Robbins et al., 2017).

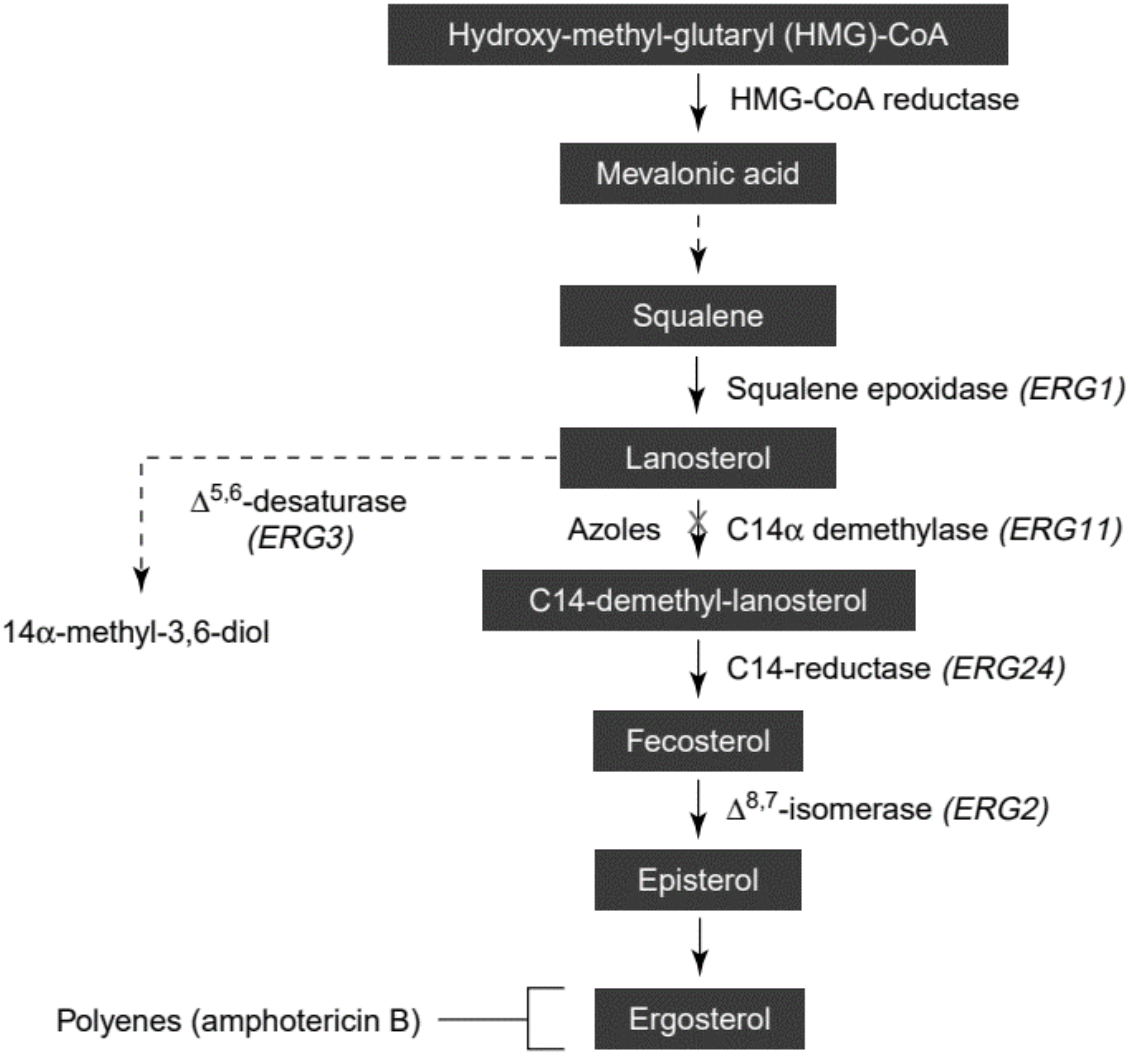


Figure 3: Ergosterol biosynthetic pathway and the action of antifungal drugs. The target enzymes are reported on the right with encoding genes in parentheses, whereas the antifungal drugs are reported on the left of the arrows indicating the sequential steps of sterol biosynthesis. From (Lupetti et al., 2002).

1.4 What is antifungal drug resistance?

The acquisition of drug resistance in pathogenic microorganisms is an evolutionary process resulting from exposure to antimicrobial agents (Cowen et al., 2002). From a clinical perspective, drug resistance can be defined as the persistence or progression of an infection despite appropriate drug therapy. Often, clinical failure may be due to low levels of the drug in patient's serum, or the presence of a severely immunosuppressive state, such as in AIDS patients, in this case, antifungal agents alone are unable to eliminate the fungi from the host (Fidel et al., 1999).

From a laboratory perspective, drug resistance is quantified by using a Minimal Inhibitory Concentration (MIC) assay, following the standards approved by the National Committee for Clinical Laboratory Standards (NCCLS) protocol for antifungal susceptibility testing of yeasts (Rex et al., 2001). In a MIC assay the growth of the pathogen is measured with a series of drug concentrations over a defined period according to standard protocols (CLSI, 2012). The lowest concentration of a drug that inhibits growth, generally by either 50% or 80%, is defined as the MIC (CLSI, 2012). Pathogens showing a higher MIC of a drug are assumed to have a better fitness in the presence of the drug than those with a lower (Cowen et al., 2002) MIC. Despite the logic of this expectation MIC is not always predictive of growth at high drug concentration. There are cases of high fitness in the presence of the drug with low MIC or low fitness with high MIC (Cowen, 2001).

In vitro resistance can be subdivided into primary resistance and secondary resistance. Primary resistance is considered when an organism is naturally resistant to the antifungal agent. Secondary resistance occurs when an organism becomes resistant to the antifungal agent (Fidel et al., 1999). This form of resistance is frequently reported in AIDS patients receiving prolonged antifungal therapy (Fan-Havard et al., 1991; Johnson et al., 1995). In *Candida* species, antifungal resistance practically had not been reported until the appearance of HIV. Indeed, in AIDS patients resistant populations emerged with the selective pressure of several antifungal agents (Fidel et al., 1999).

When compared to other *Candida* species, *C. glabrata* isolates usually have higher MICs (Shapiro et al., 2011)) for all azoles and are naturally less susceptible to all antifungal agents (Fidel et al., 1999). *C. glabrata* possesses intrinsically low susceptibility to fluconazole, the MIC of this drug in *C. glabrata* being approximately 16 times higher than that for *C. albicans* (Pfaller et al., 2002). Although, primary in vitro resistance to fluconazole has been reported in *C. glabrata* (Rex et al., 1995a; Rex et al., 1995b), secondary in vitro resistance is the most common form of resistance in *C. glabrata* (Wingard, 1994, 1995; Wingard et al., 1993) and is very common for fluconazole (Fidel et al., 1999). The reason for this rapid development of secondary antifungal resistance is

not clear, but it is thought that the haploid genome of *C. glabrata* may be a contributing factor (Fidel et al., 1999).

Understanding the molecular mechanisms that lead to clinical resistance is critical and may be reached through the identification of the molecular alterations in the genome of strains that evolved from susceptible towards an azole resistance phenotype.

1.5 Azole resistance

1.5.1 Adaptive mechanisms of resistance to azoles

Fungi can adapt mechanisms by which antimicrobial resistance evolves, contributing to the acquisition of drug resistance in clinical isolates (Robbins et al., 2017)**.** The natural capacity to evade the immune system by proliferating inside macrophages exhibited by *C. glabrata* may be a key factor in the capability of this species to persist during multiple antifungal treatments and develop multidrug resistance (Rodrigues et al., 2014). There are 4 possible mechanisms reported for resistance to azoles:

1.5.1.1 - Decreased affinity of azoles to cellular target:

The alteration of the azole target on the cell is a possible mechanism that reduces the action of the drug. As already mentioned, azoles have Erg11 as cellular target, therefore, individual mutations in *ERG11* can confer azole resistance affecting the affinity to the drug (Sanglard et al., 1998; Shapiro et al., 2011). In 2000, Ji et al. developed a 3D model of the structure of *C. albicans* Erg11, predicting important residues for its interaction with azoles. (Ji et al., 2000). Subsequently 12 different point mutations in Erg11, clustering into three distinct “hot-spot” regions adjacent to the enzyme active site, were associated with azole resistance in *C. albicans* clinical isolates (Shapiro et al., 2011). Mutations in homologous genes have also been reported to confer azole resistance in *C. neoformans* (Rodero et al., 2003) and *A. fumigatus* (Diaz-Guerra et al., 2003; Mann et al., 2003). In addition, in *C. krusei*, intrinsic resistance to fluconazole has been reported and related to a mechanism associated with reduced affinity of Erg11 to azoles (Orozco et al., 1998). On the other hand, in C.*glabrata ERG11* does not appear to have an important role in clinical azole resistance (Sanguinetti et al., 2005; Szweda et al., 2015; Vermitsky and Edlind, 2004). Until today no clinical isolates have been confirmed to have resistance mechanisms related to a decreased affinity of azoles to cellular target in *C. glabrata* (Whaley et al., 2016).

Related studies demonstrated that many *Candida* species display increased fluconazole susceptibility under iron-limited conditions (Prasad et al., 2006). Furthermore, it was demonstrated that iron depletion leads to down-regulation of *ERG11* and consequently a partial

failure in ergosterol biosynthesis, resulting in an increase in membrane fluidity and in passive drug diffusion in *C. albicans* cells (Prasad et al., 2006). Hosogaya et al. revealed that *C. glabrata* also exhibits increased azole susceptibility under low-iron conditions. In this study, the disruption of *DAP1*, encoding a heme-binding protein involved in ergosterol biosynthesis through the activation of Erg11, resulted in growth defects under iron-limited conditions, decreased azole tolerance, decreased production of ergosterol. Interestingly, the tolerance phenotype was rescued by exogenous ergosterol and not by iron supplementation alone. *DAP1* seems, thus, to be crucial for Erg11 activity and ergosterol biosynthesis and, hence, required for azole tolerance (Hosogaya et al., 2013).

1.5.1.2 - Azole cellular target Overexpression:

Another possible mechanism involves the overexpression of the drug target Erg11 (Robbins et al., 2017). In *C. albicans* the transcriptional activator Upc2 was demonstrated to regulate the expression of *ERG11* and other genes involved in ergosterol biosynthesis, upon exposure to azoles. Gain-of-function mutations in the transcription activator Upc2 may lead to constitutive overexpression of *ERG11* in azole-resistant isolates (Dunkel et al., 2008; Hoot et al., 2011; Morio et al., 2013). In fact, *UPC2* disruption results in hypersusceptibility to azoles and reduced accumulation of exogenous sterols. Additionally, the introduction of the mutated allele into a drug-susceptible strain resulted in constitutive upregulation of *ERG11* and increased resistance to fluconazole (Dunkel et al., 2008). Another mechanism involving genomic alterations (mitotic recombination, gene conversion, and the formation of an isochromosome) that amplify the copy number of *ERG11* was also reported in *C. albicans* (Selmecki et al., 2006; Selmecki et al., 2008). Mechanisms involving *ERG11* overexpression were also observed in *C. neoformans* and *A. fumigatus* (Robbins et al., 2017). Overexpression of *ERG11* in *C. glabrata* has been observed in two clinical isolates (Redding et al., 2003; vanden Bossche et al., 1992). However, one was later found to be due to duplication of the entire chromosome containing *ERG11*, while the other, the phenotype was lost with subsequent passaging in azole-free media (Whaley et al., 2016), suggesting that this is not a usual mechanism of azole resistance acquisition in the clinical setting.

1.5.1.3 - Alterations in ergosterol biosynthetic pathway:

When azoles binds to the iron atom in the heme group located in the active site of Erg11, blocking the production of ergosterol, resulting in accumulation of 14-α-methyl-3,6-diol, toxic sterol produced by the Δ-5,6-desaturase encoded by *ERG3*, the fungal cells can develop an altered ergosterol biosynthetic pathway in order to deal with this toxicity (Lupetti et al., 2002). This alteration has been linked to point mutations that lead to the inactivation of the Erg3 enzyme, leading to the accumulation of 14α-methylfecosterol. This event partially overcomes the lack of ergosterol in the plasma membrane, but especially prevents the synthesis and accumulation of toxic sterols. The altered sterol, 14α-methylfecosterol, allows continued growth and the production of functional membranes which are otherwise disrupted by the polar 6-OH group of the diol (Lupetti et al., 2002). This mechanism can provide also cross-resistance to antifungal amphotericin B (Kelly et al., 1997). These alterations in ergosterol biosynthetic pathway were reported in *C. albicans*, however, in *C. glabrata* they result in an altered sterol composition of the membrane but do not confer fluconazole resistance (Kelly et al., 1997; Martel et al., 2010).

1.5.1.4 - Reducing intracellular azole accumulation:

Another way to evolve resistance to azoles is by reducing accumulated intracellular drug by activation of efflux pumps (Robbins et al., 2017). Upregulation of the expression of either of two classes of multidrug transporters, ATP-binding-cassette (ABC) or major facilitator superfamily (MFS), is a very common mechanism responsible for azole drug resistance in *Candida* species (Cowen and Steinbach, 2008). In fact, up-regulation of drug efflux pumps from the ABC superfamily has been found to be a major contributor mechanism of azole resistance in *C. glabrata* (Tsai et al., 2010). In *C. glabrata*, this mechanism is mediated by upregulation of a single or a combination of ABC transporters, among which Cdr1, Pdh1, Yor1, Snq2, that contribute to xenobiotic drug efflux (Noble et al., 2013; Rodrigues et al., 2014; Vale-Silva et al., 2013). The expression of *C. glabrata* ABC transporter genes are regulated by a main transcriptional regulator of pleiotropic drug resistance (Noble et al., 2013; Vermitsky and Edlind, 2004), CgPdr1, that belongs to the family of zinc finger transcription factors with Zn(2)Cys(6) domains (Vermitsky and Edlind, 2004), and plays a central role in fluconazole resistance acquired by *C. glabrata* (Ferrari et al., 2009; Tsai et al., 2006; Vermitsky and Edlind, 2004). CgPdr1 regulates other genes in *C. glabrata* due to the presence of a PDRE (pleiotropic drug response element) in the promoters of its target genes (Ferrari et al., 2009). CgPdr1 appears to also control the regulation of its own transcription, as suggested by the presence of a PDRE in its promoter (Ferrari et al., 2009). Upregulation of ABC transporter genes in azole-resistant strains is correlated with an increase of *CgPDR1* expression, reinforcing this idea (Vermitsky et al., 2006). In Thakur et al., 2008, CgPdr1

was shown to act as nuclear receptor, binding directly to azoles and other drugs. Then, the activation domain of CgPdr1 binds directly to the mediator co-activator subunit CgGal11 in a xenobiotic-dependent manner. This will lead to the activation of transcription of target genes, like efflux pumps genes, resulting in multidrug resistance (Thakur et al., 2008). The transcription factor *CgSTB5* was found to be a negative regulator of azole resistance and acts as transcriptional repressor of ABC transporter genes. Expression analysis found that CgStb5 shares many transcriptional targets with CgPdr1 but is a negative regulator of pleiotropic drug resistance. Its overexpression represses azole resistance in *C.glabrata*, and its deletion causes a slight increase in resistance (Noble et al., 2013).

In fact, acquisition of azole resistance in clinical isolates of *C. glabrata* has been almost exclusively linked to the transcriptional upregulation of multidrug transporter genes, resulting from mutations in the zinc cluster transcription factor Pdr1, called gain-of-function (GOF) mutations (Tsai et al., 2010). Several amino acid substitutions in CgPdr1 have been reported as GOF (table 3) and to increase the expression of at least *CgCDR1*, *CgPDH1* and *CgSNQ2* and thus to contribute to azole resistance of clinical isolates (Ferrari et al., 2009; Ferrari et al., 2011b; Gohar et al., 2017; Ni et al., 2018; Tsai et al., 2006; Tsai et al., 2010; Vale-Silva et al., 2013). It is unclear why *C. glabrata* is capable of rapid acquisition of *PDR1* mutations, but is thought to be due, at least partially, to the high incidence of loss-of-function mutations in the mismatch repair gene *MSH2*, which results in a hypermutable phenotype (Healey et al., 2016b). Despite GOF mutations in *PDR1* are the main reported mechanism of resistance acquisition in *C. glabrata*, some studies have shown that there are cases of clinical isolates with resistance to fluconazole without *PDR1* mutations (Hou et al., 2018; Katiyar et al., 2016; Tian et al., 2018; Vermitsky and Edlind, 2004).

Upregulation of ABC transporter genes can also occur without GOF mutations but in mutants with mitochondrial defects, so-called petite-mutants (Ferrari et al., 2009). These mutants have mitochondrial DNA deficiency resulting in a respiratory-deficient cell with nonfunctional mitochondria and were shown to be resistant to azoles with both *CgCDR1*, *CgPDH1* and *CgSNQ2* upregulated (Ferrari et al., 2011a; Rodrigues et al., 2014; Sanglard et al., 2001). The azole resistance of these petite mutants is dependent on *CgPDR1* (Tsai et al., 2006), but the exact regulatory mechanisms behind the upregulation of ABC transporters in petite mutants are still largely unresolved (Ferrari et al., 2011a). Additionally, petite mutants exhibit altered sterol profiles, but without changes in the sequence or expression of *ERG11* (Brun et al., 2004). These mutants can be obtained in the laboratory by treatment with azoles or ethidium bromide (Sanglard et al., 2001). Although *C. glabrata* petite mutants have been recovered directly from patient samples (Bouchara et al., 2000), they are not commonly found among clinical isolates (Whaley et al., 2016).

Table 3: Reported GOF mutations in CgPdr1 (Caudle et al., 2011; Ferrari et al., 2009; Ferrari et al., 2011b; Gohar et al., 2017; Ni et al., 2018; Salazar et al., 2018; Tsai et al., 2006; Tsai et al., 2010; Vale-Silva et al., 2013).

L139I	E340D	E555K	R772K	G943S
D243N	S343F	F575L	R772I	L946S
D261G	L344S	H576Y	V785D	F948I
K274N	G346V	F580C	I803T	M957I
K274Q	G346D	G583S	F817S	N1077D
L280F	L347H	G583D	P822L	G1079V
K284N	G348C	Y584C	F853S	G1079R
Y285C	G348A	T588A	F859L	T1080A
L291R	T360I	R592S	D876Y	T1080I
R293I	Y372N	R592G	D876G	D1082G
W297R	I373V	T607S	T885A	E1083Q
W297S	R376W	T607A	L890F	D1089Y
W316L	R376G	N691D	P915R	L1093P
S316I	R376Q	N764D	P927S	G1099A
L328F	S391L	N764I	S942P	G1099D
G334E				

Recently, up-regulation of four MFS transporters, CgQdr2, CgTpo1_1, CgAqr1 and CgTpo3, has been demonstrated to occur in clotrimazole resistant isolates of *C. glabrata* compared to the susceptible isolates (Costa et al., 2016). The deletion of one of these genes, *CgTPO3*, was found to cause a moderate increase in susceptibility to clotrimazole and fluconazole and an increase in accumulation of azole drugs. These findings suggest MFS transporters may have an involvement in the clinical manifestation of azole resistance in *C. glabrata* (Costa et al., 2016). In fact, it was demonstrated that Mdr1, a MFS transporter frequently expressed at non-detectable levels in wild-type *C. albicans* strains, is constitutively overexpressed in some fluconazole-resistant *C. albicans* isolates (Alarco and Raymond, 1999). CaMrr1, a zinc cluster transcription factor, is upregulated in fluconazole resistant *C. albicans* isolates and is considered as the central regulator of the Mdr1 efflux pump in this yeast (Morschhauser et al., 2007). Mrr1 binds to elements in the promoter region of *MDR1* resulting in an overexpression of the efflux pump in drug-resistant clinical strains (Schubert et al., 2011; Vandeputte et al., 2012).

1.5.2 Biofilms

Microorganisms can exist in nature as biofilms, structurally and functionally complex communities enclosed in a self-produced polymeric matrix attached to surfaces instead of isolated free-floating planktonic organisms (Costerton et al., 1999; Rodrigues et al., 2014). Biofilm cells are much more resistant to antimicrobials than planktonic cells and it is estimated that they are involved in 65 % of nosocomial infections (Mah and O'Toole, 2001). In addition to the four possible mechanisms mentioned above, *C. glabrata* has the ability to form biofilms, which may play an important role in drug resistance, independently of defined genetic alterations. Different factors may be responsible for the intrinsic resistance of *C. glabrata* biofilms (Rodrigues et al., 2014). The cell density is considered an important factor for antifungal resistance in biofilms, especially for azoles (Rodrigues et al., 2014). In dense biofilms, cells can communicate and coordinate their behavior through quorum sensing, a process that allows cooperation between individual cells, enabling the secretion of signaling molecules, which are dependent on the population density (Ramage et al., 2012). Another important factor is the biofilm matrix. It is thought that the matrix reduces the penetration of drugs, like antifungals, through the formation of a barrier to drug diffusion (Douglas, 2003). Extracellular polysaccharides are key elements of the biofilm matrix of many microorganisms. In fact, a very important polysaccharide in *C. albicans* has been associated to biofilm protection from fluconazole (Taff et al., 2012). Another factor is the activation of stress response genes in biofilm cells (Seneviratne et al., 2010). Persister cells, dormant variants of normal cells highly tolerant to antimicrobial agents, can also be an important mechanism of resistance in fungal biofilms (Rodrigues et al., 2014).

1.5.3 Adhesins

Adhesins are specific cell-wall proteins involved in the adhesion of *Candida* species to biotic or abiotic surfaces (de Groot et al., 2008). Fungal adhesins share a common three-domain structure. C-terminal part which contains a glycosylphosphatidylinositol (GPI)-anchor, allowing the link of the adhesins to the cell wall; the N-terminal part which contain a signal sequence to target the protein to the endoplasmic reticulum; and the middle domain, characterized by the presence of multiple serine/threonine-rich repeats (Timmermans et al., 2018; Verstrepen and Klis, 2006). Despite this structural similarity, there is a wide variety of adhesins (Verstrepen and Klis, 2006). *C. glabrata* has a high number of predicted adhesins encoded by its genome, 67, many of them confirmed to be involved in adherence to a specific substrate (de Groot et al., 2008; Weig et al.,

2004). Since *Candida glabrata* naturally lacks hyphal growth, adhesins may play an important role in virulence and biofilm formation (Timmermans et al., 2018). In fact, there is a large number of adhesin-like encoding genes present in *C. glabrata* clinical isolates reinforcing that adhesins play an important role in infection (Timmermans et al., 2018). EPA gene family is the major group of adhesins in *C. glabrata*. The expressions of these genes, which is induced by the presence of nicotinic acid, seems to be responsible for the high ability of *C. glabrata* to adhere to different surfaces (Ma et al., 2007). *EPA1* was the first adhesin described in *C. glabrata* as being involved in adhesion, this adhesin is important for adhesion to epithelial and human macrophage-like cells (Cormack et al., 1999; Kuhn and Vyas, 2012). Interestingly, *EPA1* was shown to be regulated by the transcription factor Pdr1 (Vale-Silva et al., 2016). Given that Pdr1 is a main transcriptional regulator of pleiotropic drug resistance, adhesins may perhaps also be involved in azole resistance.

1.5.4 Other Mechanisms

Genomic plasticity is a mechanism for adaptation to environmental signals such as antifungal drug pressure, and can be an advantage in the human body, where environmental conditions fluctuate a lot (Bader et al., 2012). The acquisition of aneuploidies for example facilitates the emergence of resistance to antifungal drugs due to an increased dosage of specific resistant determinants (Robbins et al., 2017). The formation of aneuploidies in response to fluconazole was reported in *C. albicans* (Harrison et al., 2014). Genomic plasticity is also present in *Candida glabrata*. In Bader et al. 2012, chromosomal aberrations and functional adaptations were shown to occur in *C. glabrata* during infection, under antimicrobial therapy and also under laboratory conditions without extreme selective pressures (Bader et al., 2012). In a previous study genome plasticity was also connected with antifungal drug resistance (Polakova et al., 2009). Analysis of *C. glabrata* clinical isolates from patients with systemic infection, indicates that this yeast undergoes drastic genome rearrangements with multiple chromosomal translocations and appearance of new chromosomes resulting in mutant strains that can have increased fitness in a certain patient environment (Ahmad et al., 2013).

The aldo-keto-reductase superfamily (AKR) comprises several proteins that catalyze the reduction of aldehydes and ketones to their corresponding alcohol products by reducing nicotinamide adenine dinucleotide phosphate cofactor (Mindnich and Penning, 2009). Several drugs are converted to reactive carbonyls and an important role of AKRs is preventing carbonyl toxicity. Several studies have demonstrated that aldo-keto-reductases are active in stress conditions, playing a role in oxidative defense, transcriptional regulation, and drug metabolism (Barski et al., 2008). In Farahyar et al. 2013 was demonstrated that the upregulation of AKR

genes is involved with increased fluconazole and itraconazole resistance in *C. glabrata* suggesting that upregulation of this gene might give a new insight into the mechanism of azole resistance (Farahyar et al., 2013).

RPN4, an important gene involved in resistance to fluconazole, is regulated by Pdr1 (Ma and Liu, 2010). Under normal and stress conditions, proteasomes seem to control various basic cellular processes. Rpn4 is a transcription factor that stimulates expression of proteasome genes by interacting with a proteasome-associated control element (Karpov et al., 2017). This gene also regulates several other genes, namely genes involved in the response to stress and genes involved in DNA repair (Spasskaya et al., 2014). In fact, the inhibition of Rpn4 in *S. cerevisiae* was demonstrated to lead to a predisposition of the cells to DNA damage (Ju et al., 2010). The underlying mechanism of action of this gene is not yet explained. Rpn4 is considered an important stress-responsive mediator, and its degradation and availability are critical for cell survival under stressed conditions (Wang et al., 2010). This gene was shown to be upregulated in several fluconazole resistant *C. glabrata* clinical isolates (Tsai et al., 2010).

1.5.4 Heteroresistance in *C. glabrata*

A phenomenon known as heteroresistance (HR), referring to the occurrence of variable cellular responses to a drug within the same population, has been described for various microbial pathogens (Ben-Ami et al., 2016; El-Halfawy and Valvano, 2015). HR can be considered a manifestation known as bet hedging (Ben-Ami et al., 2016), whereby genetically identical cells express different phenotypic profiles, thus, maximizing the population long-term fitness during stressful fluctuations of environment, distributing risk among individuals (Levy et al., 2012).

For the determination of drug resistance there are standard testing methods, such as Minimal Inhibitory Concentration (MIC) determination and disc diffusion assays. However, heteroresistance is a poorly characterized phenomenon, and consensus-based standards to define it are lacking (El-Halfawy and Valvano, 2015). This lack of characterization prevents establishment of its clinical significance and implementation of proper therapeutic interventions and guidelines (El-Halfawy and Valvano, 2015). In fact, heteroresistance may have an important clinical significance, since the emergence of drug resistance during treatment may imply the existence of nonsusceptible subpopulations within a predominantly susceptible isogenic microbial population (Ben-Ami et al., 2016). Thus, HR is generally not detected in standard susceptibility assays, and this failure may result in misclassification of nonsusceptible strains as susceptible and implementation of wrong therapeutic procedures (Ben-Ami et al., 2016). Indeed, when heteroresistant strains are cultured in serial passages on media containing inhibitory

concentrations of an antimicrobial drug, in each generation the nonsusceptible subpopulation gradually expands and fully resistant colonies will ultimately emerge (Ben-Ami et al., 2016).

Among the different methods for measuring heteroresistance, population analysis profiling (PAP) is considered the gold standard (El-Halfawy and Valvano, 2015). In this method, the population is subjected to a gradient of drug concentrations, and the growth at each of these concentrations is quantified. PAP is typically performed using the format of standard MIC determination, with 2-fold drug increments, and by use of spread plate techniques for CFU counting (El-Halfawy and Valvano, 2015). Disc diffusion, E-test assays and flow cytometry are other possible methods (El-Halfawy and Valvano, 2015).

Heteroresistence can be defined in binary terms as a minimal fold difference between the lowest drug concentration associated with maximal growth inhibition and the highest noninhibitory concentration referred as the heterogeneity range. Heteroresistance is considered when drug concentration exhibiting the highest inhibitory effects was at least 8-fold higher than the highest noninhibitory concentration (El-Halfawy and Valvano, 2013). The use of this classification can led to confounding observations, since most studies lacked criteria to define homogeneous versus heterogeneous resistance (El-Halfawy and Valvano, 2015). To determine a meaningful fold difference breakpoint for heteroresistence, a modified PAP assay (Wootton et al., 2001) comparing the area under the curve (PAP-AUC) (figure 4) of a given strain to that of a reference heteroresistant strain can be used, providing a continuous measurement of heterogeneity (Ben-Ami et al., 2016).

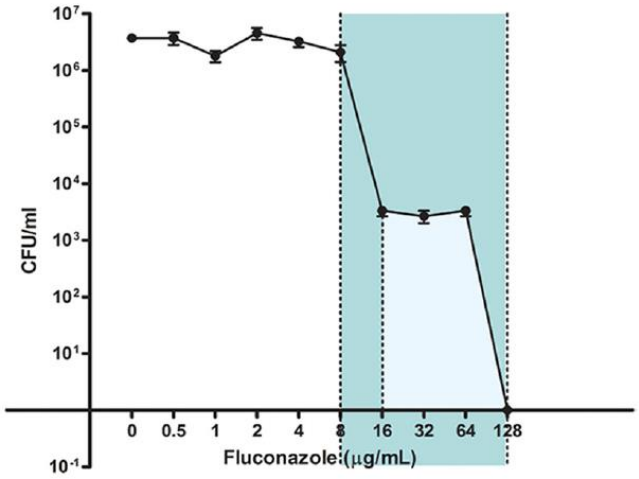


Figure 4: Representation of fluconazole population analysis. The heterogeneity range (HR) is marked in dark blue, and the AUC is marked in light blue (Ben-Ami et al., 2016).

This variation verified in heteroresistance within a population can be attributed to genetic, epigenetic, and nongenetic mechanisms. Genetic mechanisms can explain many cases of variation within a population. Mutation or duplication of key resistance genes or regulatory systems can lead to an increase in antifungal resistance and, in a long-term infection, to genomic instability, leading to heteroresistance. Epigenetic variation across the population can also occur when one or more genes whose products are involved in resistance to drugs are differentially expressed among cells within a population. Nongenetic mechanisms involved in heteroresistance include the heterogeneous presence of chemicals in the environment that may modulate the response to antibiotics across the population (El-Halfawy and Valvano, 2015).

Ben-Ami et al. 2016 described for the first time heteroresistance to fluconazole in *C. glabrata*, a finding that may explain the propensity of this pathogen to acquire resistance following exposure to fluconazole, that persists beyond treatment. Heteroresistance was found to be a continuously distributed phenotype associated with increased expression of genes that encode ABC transporters, *CDR1* and *PDH1* (Ben-Ami et al., 2016).

1.5.4 Multidrug Resistance in *C. glabrata*

The sudden emergence of *C. glabrata* strains resistant to both triazole and echinocandin is alarming and has received great concern (Healey et al., 2016a). In fact, accord to CDC/SENTRY Antimicrobial Surveillance Program, none of the fluconazole-resistant *C. glabrata* isolates from 2001 to 2004 demonstrated echinocandin resistance, however, between 2006 and 2010 11% of fluconazole-resistant isolates were also resistant to an echinocandin (Pfaller et al., 2012). Recently, other studies have reported multidrug resistance (MDR) in *C. glabrata* (Alexander et al., 2013; Farmakiotis et al., 2014). The appearance of MDR *C. glabrata* strains is attributed to the current guidelines for prophylaxis and treatment. The majority of MDR strains have acquired resistance to triazole and echinocandin class antifungals, however, even more alarming are cases of some isolates demonstrating also resistance to amphotericin B (Cho et al., 2015; Farmakiotis et al., 2014).

The predisposition of *C. glabrata* to develop MDR may be related to its haploid genome (Healey et al., 2016a), however, defects in DNA repair may also have an important role in this mechanism (Healey et al., 2016a). In fact, it was recently discovered that 55% of all *C. glabrata* isolates recovered from patients contain mutations within the mismatch repair gene, *MSH2*, a gene responsible for mechanisms dedicated to repairing DNA damage. Defects in these mechanisms are frequently related with increased mutation rates or genome rearrangements (Healey et al., 2016b). Furthermore, a higher frequency of emergence of resistance in vitro was verified in strains with specific *MSH2* mutations (Healey et al., 2016a). Another factor related to this predisposition

of *C. glabrata*, is its genomic plasticity (Polakova et al., 2009). Surprisingly, *C. glabrata* seems to tolerate a high degree of mutations and genome rearrangement and evolve under these circumstances (Healey et al., 2016a).

1.6 Microevolution

C. glabrata strains can develop resistance to fluconazole in vitro, in less than four days of continuous culture with low doses of the drug (Borst et al., 2005). The same can be observed in vivo in patients treated with azoles for longer periods (Mann et al., 2009; Sanglard et al., 2001). The main driving force for this phenomenon is known as microevolution (Brunke et al., 2014). Microevolution is an evolutionary process within populations that occurs in days or weeks and generates new variants of a given species or subspecies (Morschhauser et al., 2000). The line which separates microevolution from what is called macroevolution is ambiguously defined by the process of speciation (Garland and Rose, 2009). Macroevolution is considered an evolutionary process that occurs within a longer period of time and, eventually, leads to the formation of new species or subspecies (Morschhauser et al., 2000). Variability generators such the accumulation of point mutations, genetic rearrangements, and the acquisition of new material by horizontal gene transfer play a key role in microevolution (Morschhauser et al., 2000).

Point mutations occur randomly throughout the genome and are the result of replication errors or incorrect repair following DNA damage (Morschhauser et al., 2000). Most of the mutations are silent, however, some may result in an amino-acid exchange causing a modification in a protein (Morschhauser et al., 2000). Furthermore, some mutations can occur in regulatory regions, thereby altering the expression pattern of a specific gene, or group of genes (Morschhauser et al., 2000). It is very common that a single point mutation affects one specific trait that may confer an advantage in a fluctuating environment (Musser, 1995). Thus, point mutations can generate new variants of a given species within relatively short periods of time, being considered an important mechanism of microevolution (Morschhauser et al., 2000). A genetic rearrangement is considered when a microorganism alters its genome also by rearrangement of existing parts, such as, gene amplifications, gene duplications, gene recombination's or loss of parts of the genome (Morschhauser et al., 2000). For example, a gene amplification may result in an increase of the production of the corresponding gene product, and this increase would be of advantage in an environment that demands its constitutive overexpression (Morschhauser et al., 2000).

A last possible mechanism that generates variability is gene transfer, although not very common in fungi (Fitzpatrick, 2012). Microorganisms may alter their characteristics very quickly by acquisition of new genetic material from other organisms. Gene transfer can occur by transformation, transduction, and conjugation (Morschhauser et al., 2000). Microevolution allows pathogens to adjust to the highest level of effectiveness to a certain environment (Maurelli, 2007).

A good example of microevolution is the development of drug-resistance in pathogenic microbes during antimicrobial therapy. Infections by *candida* species are commonly treated with the antifungal drug fluconazole. Fluconazole is a fungistatic drug that inhibits growth, but does not kill the fungus, providing the opportunity for resistance development. For diverse pathogens, such small-scale evolution has been shown to occur during infections. Studding of microevolution is really important to understand the pathogenesis of infectious diseases (Brunke et al., 2014; Morschhauser et al., 2000).

1.7 Experimentally driving evolution towards drug resistance

Using clinical samples of pathogenic populations, sometimes does not allow a great understanding of the dynamics and genetic mechanisms of the evolution of antifungal resistance. For this reason, experimental evolution of fungal pathogens in the laboratory is often used (Cowen et al., 2002). With experimental evolution it is possible to replicate experiments and to control conditions such as ploidy, size of population, strength of selection, rate of mutation, and opportunity for genetic exchange and recombination (Cowen et al., 2002). This type of experiments allows a fungal pathogen to evolve in the presence of an antifungal drug in the laboratory, and the genotypic and phenotypic changes in the evolved populations can be compared to the initial strain (Cowen et al., 2002). Experimental evolution typically begins with one known genotype and then follows a trajectory of change over time. Using experimental populations from a single genotype prevents immigration of genotypes and genetic exchange between individuals, thus, mutation is the only source of genetic variation. This allows the identification of the specific genetic changes that underlie adaptation and the temporal sequence in which they occur relative to the known ancestral genotype (Cowen et al., 2002). The evolutionary trajectory of an experimental population depends on the availability of mutations, and this can be affected by two important parameters, the mutation rate, and the population size. Given a constant mutation rate, population size will be the most important factor of adaptation. (Cowen et al., 2002).

In large populations the effect of natural selection becomes more evident (Wahl and Krakauer, 2000), because in smaller populations genetic drift may overlap with selection, with some genotypes increasing in frequency by chance, and not by their adaptive fitness (Cowen et al., 2002). For this reason, large experimental population sizes may be more appropriate for studying adaptation. However, real pathogen populations are often bottlenecked to very small sizes during transmission between hosts. In fact, within the entire population infecting one host only a small fraction is transmitted and successfully colonizes a new host (Bergstrom et al., 1999). Additionally,

in this bottlenecked population, the best-adapted genotypes are not necessarily transmitted to a new host, especially if these genotypes are at a low frequency in the population (Levin et al., 2000).

When running an evolution experiment there are some important conditions that must be considered. Drug concentrations can´t be very high, must be adjusted to substantially inhibit the growth of the fungus without resulting in extinction, the concentration will also depend on the type of action of the drug, fungicidal or fungistatic (Cowen et al., 2002). The stability of resistance can be observed by subsequent evolution in the absence of any drug (Cowen et al., 2002). Additionally, selection can occur under incremental increases in drug concentration or under one step at a high drug concentration. Depending on the adopted strategy, different mechanisms of resistance can be obtained. Using incremental steps, the pathogen has the capacity to adapt, acquiring compensatory mutations, contrary to the one step selection (Cowen et al., 2002). Another important factor is the timescale for the evolution experiment. Evolution experiments of a few hundred generations allows an approximation to a pathogen evolving drug resistance in its host during a course of drug treatment, while evolution experiments over thousands of generations allows the evaluation of whether the populations ultimately converge on one stable, adaptive optimum (Lenski and Travisano, 1994). One last important condition is the choice between an unstructured and a structured environment. In an unstructured environment, such as those found in chemostats or batch cultures with constant agitation, spatial associations between individuals are continually disrupted. In contrast, in a structured environment, physical and spatial associations between individuals are rarely disrupted. These environments can be provided by an animal model, a biofilm or even the surface of a petri dish. In the animal models it is important to consider added factors, such as the host physiology and the immune system (Cowen et al., 2000). Most experimental evolution studies have used microbial populations in liquid media, in a chemostat or in batch cultures with serial passages into fresh medium (Cowen et al., 2000; Zeyl, 2000). With an unstructured environment it is expected a succession of genotypes to increase in frequency in the population. On the other hand, structured environments may favor radiations (process in which organisms diversify rapidly from an ancestral species) if different genotypes predominate in different niches (Rainey and Travisano, 1998). Murine models are considered the gold standard animal model for fungal studies (Ames et al., 2017a). However, given the cost, legislation and ethical considerations of this model, alternative models have been explored, such as *Dictyostellium discoideum*, *Caenorhabditis elegans*, *Drosophila melanogaster* or *Galleria mellonella* (Desalermos et al., 2012). The last has received particular attention as an alternative host for *C. glabrata* as it displays some important advantages (Ames et al., 2017b).

1.8 Evolution of Resistance in *C. glabrata*

The emergence of drug resistance is present in all pathogenic microorganisms, and is considered an evolutionary process initiated by exposure to antimicrobial agents (Cowen et al., 2002). Resistance arises because antimicrobial agents rarely completely eradicates the pathogen population, and the survivors are subjected to natural selection (Cowen et al., 2002). Without complete eradication of the pathogen the evolution of resistance can be all but inevitable (Cowen et al., 2002).

The evolution of antifungal drug resistance in the pathogen proceeds on three scales: temporal scales, which may include ancient events related with speciation or also contemporary changes occurring for example within one patient under treatment (Cowen et al., 2002); spatial scales, which can range from large-scale populations of patients to fine-scale niches within the body of one patient (Cowen et al., 2002); genomic scales, which can range from the small-scale resistance mutations with one or a few genes to large-scale interactions with many genes and considerable changes in genome-wide patterns of gene expression in the pathogen (Cowen et al., 2002).

Genetic variability is the source of evolution of drug resistance, mainly through mutations (Cowen et al., 2002). However, the establishment of a mutation conferring resistance in a population depends on both the fitness effect of the mutation and on the population size, and is determined by a set of key processes: selection, genetic drift, recombination, and migration (Cowen et al., 2002). The time resistance takes to spread in a pathogen population exposed to a drug as well as its capacity to resist the absence of the drug is determined by the relative fitness (Cowen et al., 2002).

Although, the evolutionary dynamics of drug resistance and its fitness costs have been deeply studied in viruses and bacteria, in fungi they await investigation. (Cowen et al., 2001). Experimental populations of *Candida albicans* from a previous study provided the opportunity to determine the fitness costs of resistance to the antifungal drug fluconazole (Cowen et al., 2000).

It is expected that in the presence of drug, a resistant genotype will be an advantage compared to less resistant genotypes. However, if drug resistance carries a fitness cost for the pathogen, in the absence of drug these resistant genotypes may be a disadvantage compared to their sensitive counterparts, adaptation of a population to one environment may result in a decrease in fitness in another environment (Cowen et al., 2000). Assuming that, there are some strategies to control the dissemination of drug resistance by restricting the use of antimicrobial agents (Levy, 1994). However, there are some studies reporting a superior fitness of resistant strains compared to parental ones, even in the absence of drug (Bouma and Lenski, 1988; Nijhuis et al., 1999). It is thought that the costs of resistance will decline during subsequent evolution because natural

selection continues to favor genotypes with a fitness advantage, (Cowen et al., 2000) observing, for example, compensatory mutations that reduces the cost of resistance (Levin et al., 2000).

The spread of genetic mechanisms conferring antifungal drug resistance in pathogen populations can be observed using epidemiological and population-genetic studies. In population-genetic studies, samples of the pathogen from patient populations may be used, or, alternatively, through experimental evolution, using batch cultures, chemostats, or animal models (figure 5) (Cowen et al., 2002).

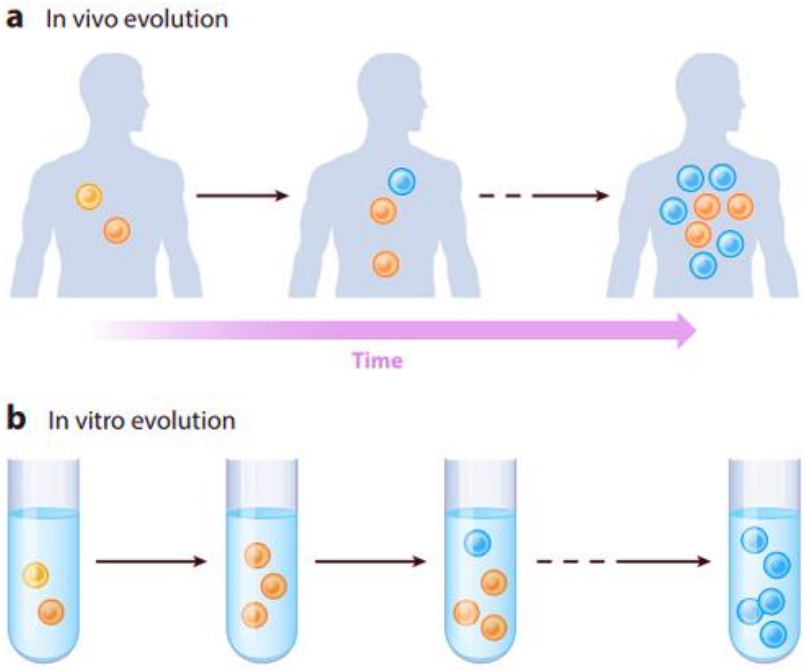


Figure 5: (a) Evolution of drug resistance in the human host. The progressive accumulation of resistance mutations in clinical isolates. Strains with a fitness advantage (blue) proliferate and outcompete other strains in the human host (yellow and orange). (b) Experimental evolution of drug resistance. Cells are passaged by serial dilution of a stationary-phase culture into fresh medium containing a concentration of antifungal that is inhibitory but not lethal. This process is repeated until a sufficient number of generations have occurred for resistance to evolve. From (Robbins et al., 2017).

Borst et al. (Borst et al., 2005) showed that despite no prior exposure to azole antifungal agents, only 2 to 4 days of in vitro exposure to fluconazole were necessary for the development of azole drug resistance in previously susceptible, naive *C. glabrata* isolates, proving that prior exposure to azole drugs is not a prerequisite for the rapid development of fluconazole resistance. In this study resistance was associated with increased expression of the ABC transporter encoding genes *CgCDR1* and *CgPDH1*, but not of *CgERG11* (Borst et al., 2005). Similar results can be

observed in echinocandin resistance, clinical isolates of *C. glabrata* can acquire resistance after 12 days of micafungin treatment without prior exposure to the drug (Sasso et al., 2017) and echinocandin susceptible isolates can acquire in vitro resistance after 2–4 days of exposure to low and constant micafungin concentrations (Bordallo-Cardona et al., 2017). In vitro evolution experiments can provide a simple model for how drug resistance emerges in the host, and these experiments are easily replicated, allow sample sizes much greater than what is achieved with clinical isolates from patients (Robbins et al., 2017). Furthermore, a global view of adaptative mutations can be performed with genome sequencing, and RNA-sequencing technology can be used for the identification of genes that are specifically overexpressed in drug-resistant isolates. Therefore, in vitro evolution constitutes a great tool for illuminating mechanisms of resistance (Robbins et al., 2017).

1.9 Research Objectives and thesis outline

Currently *Candida glabrata* is one of the most isolated non-albicans *Candida* species and the infections caused by this pathogenic yeast are associated to high mortality rates. The unusual ability to develop azole resistance in the human host makes this yeast a major clinical challenge with severe economic and health impact. It is thus pivotal to understand the molecular basis of this phenomenon.

This master's project aims to contribute to this field, by establishing new clinically relevant mechanisms of azole resistance, that go beyond the current model that is limited to the role of GOF mutations in the Pdr1 transcription factor that lead to constitutive overexpression of multidrug transporters.

This thesis starts with an Introduction that reviews current knowledge on *C. glabrata* azole resistance mechanisms and their regulation.

The second chapter details the materials and methods used during the execution of this project, while the third chapter describes the obtained results and discusses them in light of the current knowledge.

Finally, the forth chapter provides the main conclusions of this study, while point out the many perspectives that it raises and that deserve further exploitation.

2. Experimental Procedures

2.1 Strains and growth media

The *C. glabrata* strains used are described in Table 4. *C. glabrata* cells were cultivated in rich YPD medium, containing per litre: 20 g D-(+)- glucose (Merk), 20 g bacterial-peptone (LioChem) and 10 g of yeast-extract (Difco); or RPMI 1640 medium (pH 7), containing 10,4 g RPMI 1640 (Sigma), 34,5 g MOPS (Sigma) and 18 g glucose (Merck) per litre.

Table 4: *C. glabrata* strains used in this study.

Strain	Description	Source
013 clinical isolate	Fluconazole R; MIC >64 μg/ml	Hospital São João, Porto, Portugal (Costa et al., 2016)
10774 clinical isolate	Fluconazole R; MIC >64 μg/ml	Hospital de Santa Maria, Lisboa, Portugal (Costa et al., 2016)
50570 clinical isolate	Fluconazole R; MIC >64 μg/ml	Hospital de Santa Maria, Lisboa, Portugal (Costa et al., 2016)
51800 clinical isolate	Fluconazole R; MIC >64 μg/ml	Hospital de Santa Maria, Lisboa, Portugal (Costa et al., 2016)
73281 clinical isolate	Fluconazole R; MIC >64 μg/ml	Hospital de Santa Maria, Lisboa, Portugal (Costa et al., 2016)
94078 clinical isolate	Fluconazole R; MIC >64 μg/ml	Hospital de Santa Maria, Lisboa, Portugal (Costa et al., 2016)
65147 clinical isolate	Fluconazole R; MIC 64 μg/ml	Hospital de Santa Maria, Lisboa, Portugal (Costa et al., 2016)
67367 clinical isolate	Fluconazole R; MIC 64 μg/ml	Hospital de Santa Maria, Lisboa, Portugal (Costa et al., 2016)
MC273 clinical isolate	Fluconazole SDD; MIC 1 μg/ml	Hospital São João, Porto, Portugal (Costa et al., 2016)
MC 426 clinical isolate	Fluconazole SDD; MIC 1 μg/ml	Hospital São João, Porto, Portugal (Costa et al., 2016)
MC123 clinical isolate	Fluconazole SDD; MIC 0.25 μg/ml	Hospital São João, Porto, Portugal (Costa et al., 2016)
MC 125 clinical isolate	Fluconazole SDD; MIC 0.125 μg/ml	Hospital São João, Porto, Portugal (Costa et al., 2016)
MC126 clinical isolate	Fluconazole SDD; MIC 0.125 μg/ml	Hospital São João, Porto, Portugal (Costa et al., 2016)
MC127 clinical isolate	Fluconazole SDD; MIC 0.25 μg/ml	Hospital São João, Porto, Portugal (Costa et al., 2016)

KUE100	Wild-type; Fluconazole SDD; MIC 16 μg/ml	Prof. Hiroji Chibana, Medical Mycology Research Center, Chiba University, Chipa, Japan
Δtpo3	KUE100_ *Δtpo3;* Fluconazole SDD; MIC 8 μg/ml	Prof. Hiroji Chibana, Medical Mycology Research Center, Chiba University, Chipa, Japan (Costa et al., 2014)
Δrpn4	KUE100_*Δrpn4;* Fluconazole SDD; MIC 4 μg/ml	Prof. Hiroji Chibana, Medical Mycology Research Center, Chiba University, Chipa, Japan
Δpdr1	KUE100_*Δpdr1;* Fluconazole SDD; MIC 2 μg/ml	Prof. Hiroji Chibana, Medical Mycology Research Center, Chiba University, Chipa, Japan
Δmrr1	KUE100_*Δmrr1;* Fluconazole SDD; MIC 8 μg/ml	Prof. Hiroji Chibana, Medical Mycology Research Center, Chiba University, Chipa, Japan
Δpdr1	66032_*Δpdr1*; Fluconazole SDD; MIC 4 μg/ml	Thomas Edlind, Department of Microbiology and Immunology, Drexel University, College of Medicine, Philadelphia, PA, USA
KUE100_1	Wild-type; Fluconazole resistance acquired on day 2; MIC 64 μg/ml	This study
KUE100_2	Wild-type; Fluconazole resistance acquired on day 2; MIC 256 μg/ml	This study
KUE100_3	Wild-type; Fluconazole resistance acquired on day 2; MIC 128 μg/ml	This study
Δtpo3_1	KUE100_ *Δtpo3;* Fluconazole resistance acquired on day 2; MIC 128 μg/ml	This study
Δtpo3_2	KUE100_ *Δtpo3*; Fluconazole resistance acquired on day 2; MIC 128 μg/ml	This study
Δtpo3_3	KUE100_ *Δtpo3*; Fluconazole resistance acquired on day 2; MIC 128 μg/ml	This study
Δrpn4_1	KUE100_*Δrpn4*; Fluconazole resistance acquired on day 2; MIC 128 μg/ml	This study
Δrpn4_2	KUE100_*Δrpn4*; Fluconazole resistance acquired on day 2; MIC 64 μg/ml	This study

Δrpn4_3	KUE100_*Δrpn4*; Fluconazole resistance acquired on day 2; MIC 128 μg/ml	This study
Δmrr1_1	KUE100_*Δmrr1*; Fluconazole resistance acquired on day 2; MIC 128 μg/ml	This study
Δmrr1_2	KUE100_*Δmrr1*; Fluconazole resistance acquired on day 2; MIC 128 μg/ml	This study
Δmrr1_3	KUE100_*Δmrr1*; Fluconazole resistance acquired on day 2; MIC 128 μg/ml	This study
Δpdr1_1	66032_*Δpdr1*; Fluconazole resistance acquired on day 8; MIC 64 μg/ml	This study
Δpdr1_2	66032_*Δpdr1*; Fluconazole resistance acquired on day 7; MIC 64 μg/ml	This study
Δpdr1_3	66032_*Δpdr1*; Fluconazole resistance acquired on day 2; MIC 64 μg/ml	This study
Δpdr1_2fr	66032_*Δpdr1* at day 8 of fluconazole exposure*;* fluconazole resistant; MIC 64 μg/ml	This study

SSD: susceptible dose dependent; R: resistant

2.2 Evolution of resistance

C. glabrata parental strain Kchr606, derived deletion mutants *Δtpo3*, *Δrpn4*, *Δmrr1, Δpdr1* and the mutant *66032_Δpdr1* were grown overnight in YPD agar plates. Yeast cells of each strain were incubated overnight on test tubes containing 3 ml RPMI 1640 medium. Cells were reinoculated in fresh RPMI medium, supplemented with 16 μg/ml of fluconazole, to an initial OD = 0.1±0.01, and incubated at 37°C, 250 rpm, for 24h. Every 24 hours the procedure was repeated for a total of 20 days. At each passage, the remaining cells were centrifuged, resuspended in 750μl RPMI and stored at -80 °C in 750μl glycerol 86%. All evolution experiments were conducted in triplicate. Fluconazole was obtained from Sigma, prepared in dimethyl sulfoxide (Sigma), frozen at -80°C until use.

2.3 Antifungal susceptibility testing

The Minimal Inhibitory Concentration (MIC) of fluconazole was determined for Kchr606 and *Δtpo3*, *Δrpn4*, *Δmrr1*, and *66032_Δpdr1* mutant strains, every day until the resistance is acquired and at days 5,10 and 20 of exposure to 16 µg/ml of fluconazole. Prior to testing, isolates were subcultured on YPD agar plates for 24 hours at 30°C. The MICs were determined according to the M27-S4 protocol of the Clinical and Laboratory Standards Institute (CLSI, 2012). The fluconazole solutions used for the MIC determination were diluted with RPMI 1640 medium (Sigma) and buffered to pH 7.0 (Sigma). The final concentrations of the antifungal agents ranged from 0.5 to 256 µg of fluconazole/ml. The MIC endpoints were read spectrophotometrically following 24 hours of incubation with an automatic plate reader (BMG LABTECH). MIC level was defined as the lowest drug concentration that produced a 50% reduction in growth compared with that of the drug-free growth control.

2.4 DNA extraction, amplification and sequencing

The fluconazole resistance isolates O13, 10774, 44596, 50570, 51800, 73281, 94078, 65147, 67367 and the fluconazole susceptible isolates MC273, MC426, MC123, MC125, MC126, MC127 collected from patients admitted to Hospital of Santa Maria (HSM), Lisboa, and Centro Hospitalar São João (CHSJ), Porto(Costa et al., 2016), and the evolved resistant mutant strains *Δtpo3*, *Δrpn4* and *Δmrr1* (day 2 of fluconazole exposure) and the parental strain kchr606 were grown overnight in YPD agar plates at 30 ◦C. In a microcentrifuge tube (one for each isolate) containing 200µl of lysis buffer (50mM Tris, 50mM EDTA, 250mM NaCl, 10% SDS) and 100uL of glass beads 3 loopfuls of yeast cells were inserted. The tube was vortexed at max speed for 2 minutes and incubated at 65°C for 1 hour. After incubation for 2 minutes on ice the lysates were centrifuged for 15 minutes at 13000 rpm and 4°C. The supernatant was transferred to a new microcentrifuge tube and 1/10 NaAC 3M at pH 4.8 and 2 volumes of absolute ethanol were added. The tubes were kept at -20 ◦C for 30 minutes. A new centrifugation was performed for 20 minutes at 13000 rpm and 4◦C. The supernatant was discarded and 500µl ethanol 70% was added, followed by a final step of centrifugation for 8 minutes at 13000 rpm and 4◦C. The supernatant was removed, and the pellet was dried using speed-Vac (Eppendorf) for 15 minutes at 45◦C. After drying, the pellet was resuspended with 50µl ddH_2O. This procedure was performed for each isolate. DNA was stored at -20°C until further use. The genes *CgPDR1*, *CgMRR1* and *CgRPN4* were amplified from the DNA extracted by PCR using a Thermal Cycler Block (Applied Biosystems), with specific primers indicated in table 5. The reaction mix included: 10 µl HF Buffer, 1 µl deoxynucleoside triphosphates (dNTPs), 1 µl primer forward, 1 µl primer reverse, 2 µl DNA template, 2 µl $MgCl_2$, 1.5 µl DMSO, 0.5 µl Taq Phusion, 31 µl H_2O. For *CgPDR1* the used

amplification program was: 30 seconds (sec) at 98°C; 32 cycles of 10 sec 98°C, 20 sec at 56°C and 2 minutes (min) at 72°C; 7 min at 72°C. For *CgMRR1*: 30 sec at 98°C; 32 cycles of 10 sec 98°C, 20 sec at 54°C and 2 min and 30 sec at 72°C; 7 min at 72°C. For *CgRPN4*: 30 seconds at 98°C; 32 cycles of 10 sec 98°C, 20 sec at 56°C and 1 min and 30 sec at 72°C; 7 min at 72°C. The reaction products were stored at-20°C until used. In order to confirm the success of the amplification, the products were separated in a 1.3% agarose gel and detected by GelRed staining and UV illumination. The amplified genes were sequenced by outsourcing at StabVida. Isolates O13, 10774, 44596, 50570, 51800, 73281, 94078, 65147, 67367, MC273, MC426, MC123, MC125, MC126, MC127 were amplified the genes *CgPDR1*, *CgMRR1* and *CgRPN4*; and strains *Δtpo3*, *Δrpn4*, *Δmrr1* and kchr606 were amplified the *CgPDR1* gene.

Table 5: Primers used for amplification of *CgPDR1*, *CgRPN4* and *CgMRR1* by PCR

Gene	Primer	Sequence
CgPDR1	Forward	5′ - ATGCAAACATTAGAAACTACAT - 3′
	Forward	5′ - TCCGCGAAACCGCCGACATT - 3′
	Forward	5′ - CACAGGCAGTTAATGAACTC - 3′
	Forward	5′ - GGAACATTGCTGAACATGTG - 3′
	Reverse	5′ - TCACAAGTAAACATCAGAAA - 3′
CgRPN4	Forward	5′ - ATGACGTCTATAGATTTGGGAC - 3′
	Reverse	5′ - TTATGCAGTGACAAATCCGATG - 3′
CgMRR1	Forward	5′ - ATGAGTACCACTACAACAATACC - 3′
	Forward	5′ - ACGAATCAGGATCGTTAGCG - 3′
	Forward	5′ - GCTGCAGAATCCGAACAGAA - 3′
	Forward	5′ - CCGCATCCAAGATTGATTAC - 3′
	Forward	5′ - GGACATCGAGCTCTTCAATTC - 3′
	Reverse	5′ - CTACTCCCAGATTGAGCCAATG - 3′

2.5 *CDR1* expression

The evolved resistant mutant strains *Δrpn4* and *Δmrr1* (day 2 of fluconazole exposure) were selected to assess the transcript levels of *CgCDR1* by quantitative real-time PCR (qRT-PCR). Total RNA samples were obtained from cell suspensions under control conditions, in mid-exponential phase cells in the absence of drugs, harvested upon reaching an OD600nm = 0.8 through a centrifugation at 7000rpm during 7 minutes at 4◦C. The samples were resuspended in 900µL of AE buffer (50mM sodium acetate; 10mM EDTA; pH 5.3; 0.1% diethyl pyrocarbonate (DEPC); autoclaved) and transferred to 2 ml eppendorf. For total RNA extraction, to each eppendorf was added 90µL of SDS (sodium dodecyl sulfate) 10% (treated with 0.1% DEPC and autoclaved) and then vortexed for 5 seconds, followed by addition of 800µl of phenol (500mL liquified phenol equilibrated with 500 ml AE buffer, stored at 4°C) and another vortex of 5 seconds.

The samples were then allowed to incubate at 65◦C for 4 minutes and cooled down quickly in a dry ice-ethanol bath for 10 minutes. After this step a centrifugation was performed at 15000rpm for 5 minutes at 4°C. The upper phase was transferred to a new eppendorf. More 2 extractions were performed with a phenol/ chloroform mixture (400µL phenol; 400µ chloroform) and in the end one more extraction with 800µL chloroform-isoamyl alcohol (24:1), in each extraction the same conditions were used, 5 seconds vortex, 15000 rpm for 5 minutes at 4◦C. After phase separation, the upper aqueous phase is transferred to a new tube, 90µL of 3 M sodium acetate (pH 5.3, 0.1% DEPC and autoclaved) and 1mL ethanol (-20°C) are added, and, after the sample is briefly vortexed, the RNA is precipitated at -20°C for at least 20 minutes. The precipitated RNA was collected by centrifugation 15000rpm at 4°C for 20 minutes, the supernatant was removed, and the RNA pellet was washed with 700µL of 70% ethanol. Then, the pellet was dried using speed-Vac (Eppendorf) for 15 minutes at 45°C. After drying, the pellet was resuspended with 30µl ddH_2O (treated with 0.1% DEPC). Aliquots were stored at -80°C until further use. The quantification and quality of prepared RNA was evaluated using ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies). cDNA for real-time reverse transcription-PCR was synthesized from total-RNA samples by using the MultiScribeTM reverse transcriptase kit (Applied Biosystems) and the 7500 RT-PCR thermal cycler block (Applied Biosystems). The quantity of cDNA for subsequent reactions was kept around 10 ng. The subsequent RT-PCR step was carried out using SYBR green reagents. Primers for the amplification of the *CDR1* were designed using Primer Express software (Applied Biosystems) and are 5'-GCTTGCCCGCACATTGA-3' and 5'-CCTCAGGCAGAGTGTGTTCTTTC-3'. The RT-PCR was carried out using a thermal cycler block (7500 real-time PCR system; Applied Biosystems). Default parameters established by the manufacturer were used, and fluorescence was detected by the instrument and recorded in an amplification plot (7500 System SDS software; Applied Biosystems). The *CgACT1* mRNA level was used as an internal control. The relative values obtained for the wild-type strain in control conditions were set as 1, and the remaining values are presented relative to that control. Statistical analysis of the results was performed using analysis of variance, and differences were considered significant for p values < 0.05.

2.6 Determination of growth rate

Overnight cultures of 66032_*Δpdr1* at day 0 *Δpdr1_2fr* (fluconazole resistant mutant *Δpdr1_2* at day 8) were diluted to OD_{600} 0.2 and grown in YPD at 30°C with orbital shaking (250rpm) for 24 h. OD_{600} was measured at the indicated time points. Two replicates were conducted for each strain.

2.7 DNA extraction and genome sequencing

C. glabrata strain 66032_Δ*pdr1* and *Δpdr1_2fr* were grown overnight in liquid YPD medium at 30ºC with orbital shaking (250 rpm). The cultures were then inoculated in fresh YPD medium and grown until early-log phase. Genomic DNA was extracted using the NZY Microbial gDNA Isolation kit (NZYTech), according to the manufacturer's instructions. The isolated DNA was sequenced on an Illumina HiSeq X, producing 2x150bp paired-end reads. Library preparation and sequencing were carried out at Admera Health, LLC, South Plainfield, NJ, USA, using a Nextera XT library. Illumina sequencing produced 88,056,764 raw paired-end reads for the susceptible strain and 73,871,504 raw paired-end reads for the fluconazole-resistant strain. Low quality bases and adapters were removed using Trimmomatic (v0.38) (Bolger et al., 2014).Read duplicates were removed using PRINSEQ (v0.20.4) (Schmieder and Edwards, 2011). A preliminary analysis revealed a low level of read contamination. 72,763,982 high-quality reads for the susceptible strain and 60,837,244 high-quality reads for the fluconazole-resistant strain were used in the subsequent analysis.

2.8 Genome size estimation and heterozygosity

A k-mer count analysis was done using Jellyfish (v2.0) (Marcais and Kingsford, 2011) using only the first read pair. The second pair was not used in the analysis to avoid counting overlapping k-mers. A k-mer size k=21 was used heterozygosity and repeat content were estimated using GenomeScope, available at http://qb.cshl.edu/genomescope/.

2.9 Variant calling

Variant analysis was performed to ascertain variant changes between the susceptible and fluconazole-resistant strains. Read error correction was performed using Karect (v1.0) (Allam et al., 2015) to minimize the influence of sequencing errors on variant calls. Corrected reads were aligned against the reference genome (*C. glabrata* CBS138) obtained from the Candida Genome Database (http://www.candidagenome.org/) using bwa mem (v0.7.17) (Li and Durbin, 2009). Variants were called with the Genome Analysis Toolkit HaplotypeCaller (v4.0.8.1) (McKenna et al., 2010) and BCFtools (v1.9) (Narasimhan et al., 2016). Variants from both callers were filtered using BCFtools according to quality (Q>30) and depth thresholds (5<depth<2000) to remove low quality calls and minimize the occurrence of false positives. The reported variants were annotated using SnpEff (v4.3) (Cingolani et al., 2012).

3. Results and Discussion

3.1 Rapid evolution towards fluconazole resistance can be induced in *C. glabrata* cells, even in the absence of important fluconazole resistance determinants

The KChr606 wild-type strain and derived *Δtpo3*, *Δmrr1*, *Δrpn4* and *Δpdr1* mutant strains were first evaluated in terms of their relative tolerance to fluconazole. The comparison was made through standard MIC assays, and the curve relating growth inhibition to fluconazole concentration obtained.

The wild-type strain was observed to have a fluconazole MIC level of 16µg/ml when compared to MIC levels of 8µg/ml, 8µg/ml, 4µg/ml and 2µg/ml, registered for the *Δtpo3*, *Δmrr1*, *Δrpn4* and *Δpdr1* deletion mutant strains, respectively (Figures 6-9). Additionally, it was possible to observe that, for the wild-type strain, there appears to be a fixed population of around 30% of the total population that is able to grow even the highest concentrations of fluconazole tested (60mg/mL). This resilient population can be hypothesized to be a consequence of heteroresistance within the wild-type population. Interestingly, the four deletion mutants exhibit a significantly reduced heteroresistant subpopulation, to values close to 10% in the case of *Δrpn4* and *Δpdr1*. This observation led us to hypothesize that the different strains should display different abilities to acquire resistance to fluconazole, upon prolonged exposure to the drug.

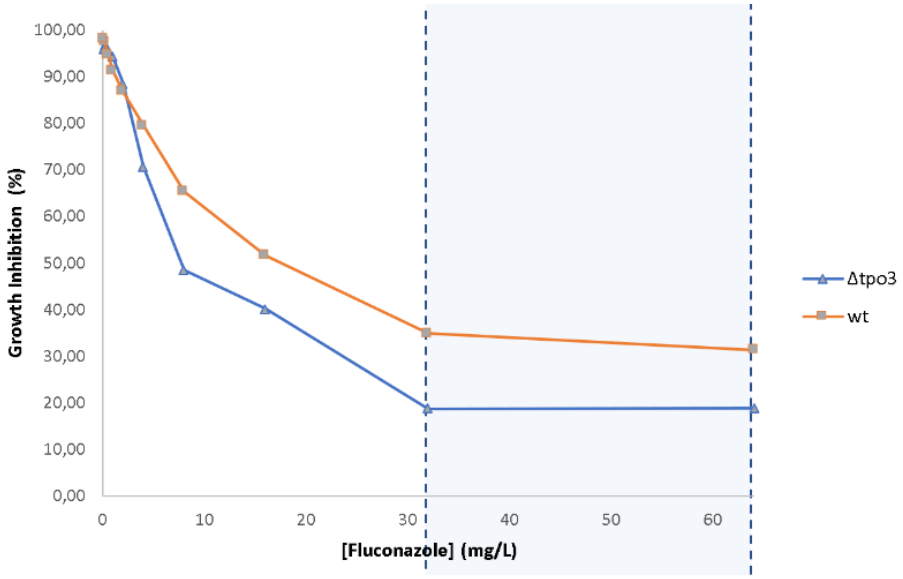


Figure 6: *C. glabrata* growth inhibition curve as a function of fluconazole concentration, obtained using the standard CLSI MIC determination assay, obtained for the wild-type KChr606 and derived *Δtpo3* strains. Between the blue area, are defined the concentrations in which the growth inhibition rate of the mutant remains the same.

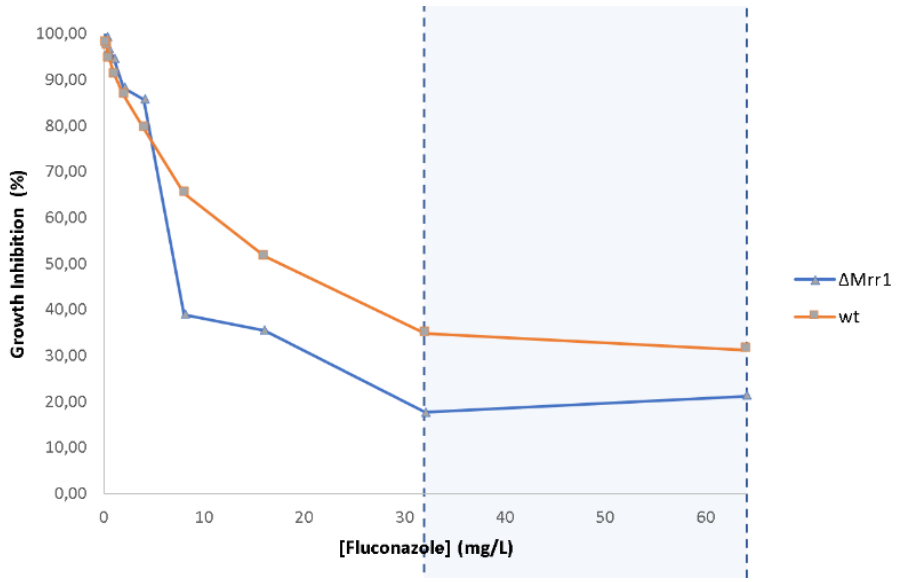


Figure 7: *C. glabrata* growth inhibition curve as a function of fluconazole concentration, obtained using the standard CLSI MIC determination assay, obtained for the wild-type KChr606 and derived *Δmrr1* strains. Between the blue area, are defined the concentrations in which the growth inhibition rate of the mutant remains the same.

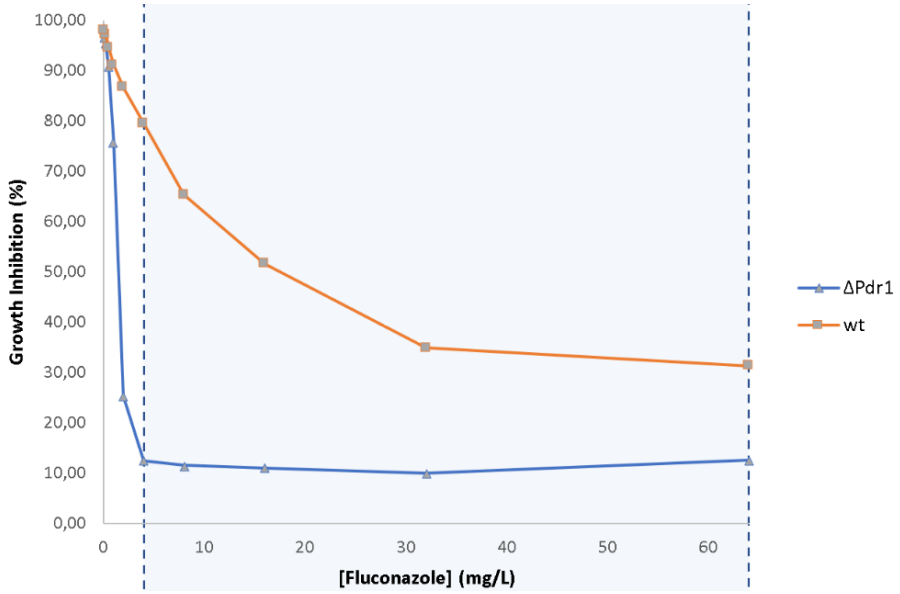


Figure 8: *C. glabrata* growth inhibition curve as a function of fluconazole concentration, obtained using the standard CLSI MIC determination assay, obtained for the wild-type KChr606 and derived *Δpdr1* strains. Between the blue area, are defined the concentrations in which the growth inhibition rate of the mutant remains the same.

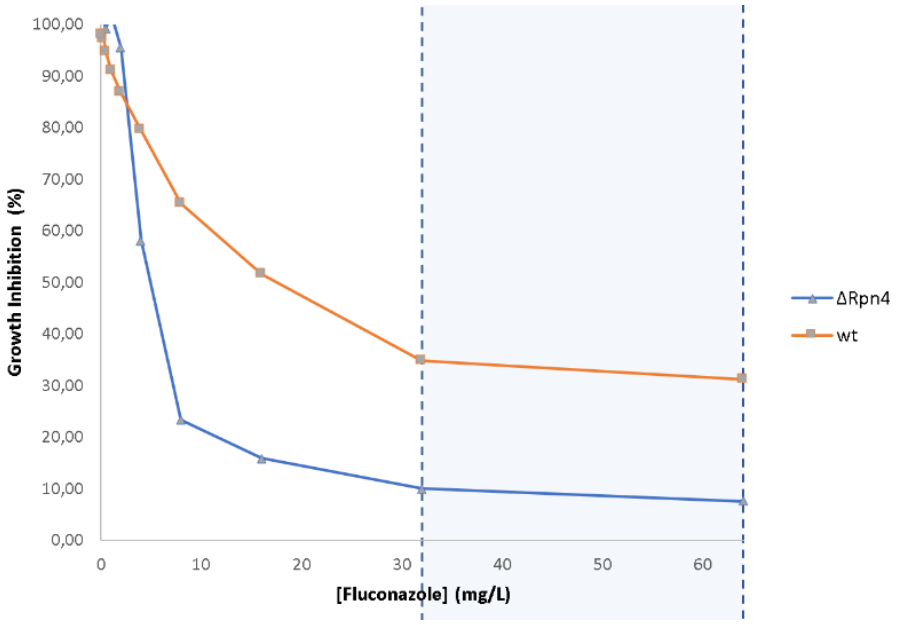


Figure 9: *C. glabrata* growth inhibition curve as a function of fluconazole concentration, obtained using the standard CLSI MIC determination assay, obtained for the wild-type KChr606 and derived *Δrpn4* strains. Between the blue area, are defined the concentrations in which the growth inhibition rate of the mutant remains the same.

To test this hypothesis, KChr606 and the derived *Δtpo3*, *Δmrr1*, *Δrpn4 and Δpdr1* strains were exposed to a constant concentration of 16μg/ml of fluconazole, considered the normal concentration of the drug in patient's serum during therapy, while conducting daily passages to fresh medium with the same drug concentration. Prior to fluconazole exposure, all *C. glabrata* strains examined were susceptible dose-dependent to fluconazole, although exhibiting varying fluconazole MIC levels (table 6). After only 2 days of exposure to 16μg/ml fluconazole, wild-type KChr606 and the mutants *Δtpo3*, *Δrpn4* and *Δmrr1* had already acquired resistance to this drug (MICs > 64 μg/ml). Indeed, *Δtpo3* and *Δmrr1* deletion mutant strains at day 2 displayed a fluconazole MIC value >128 μg/ml, while KChr606 and *Δrpn4* exhibited a fluconazole MIC level of > 64 μg/ml. The used KChr606 derived *Δpdr1* mutant was found to acquire azole resistance at day 5. However, after sequencing an unexpected PCR product, resulting from the amplification of the *PDR1* gene, it was found that the initial strain was, surprisingly contaminated with wild-type cells. We hypothesized that the initial mutant should be contaminated with a small number of wild-type cells, and with the serial passages these cells were selected by the fluconazole pressure, eventually overlapping those of the mutant. For this reason, this mutant was not considered for further analyses.

Table 6: In vitro fluconazole MIC levels of *C. glabrata* strains before and after evolution towards fluconazole resistance.

Strain	Fluconazole MIC (µg/ml)	
	Initial	1st Resistant [a]
KChr606	16	64 (day2)
Δtpo3	8	128 (day2)
Δrpn4	4	64 (day2)
Δmrr1	8	128 (day2)
Δpdr1[b]	2	64 (day 5)

[a] First resistant mutant population fluconazole MIC level to emerge during fluconazole exposure; day of culture when resistance was acquired is shown in parentheses. [b] This mutant was found to be contaminated with wild-type KChr606.

Interestingly, some heterogeneity in terms of resistance acquisition was observed in the triplicate experiments (Figure 10). It is likely that different subpopulations may evolve in different ways. Although each replica starts from the same strain, variations may also occur within the same population, this hypothesis being supported by the possible existence of heteroresistance as demonstrated. The strains were found to be capable of achieving a maximum MIC between 128 µg/ml and 256 µg/ml, thus, being able to tolerate high fluconazole concentrations, when the considerable limit for resistance is 64 µg/ml. In order to determine the mechanisms behind the resistance of these strains, the *PDR1* gene from each replicate was sequenced. Given the importance of the CgPdr1 transcription factor in fluconazole resistance in *C. glabrata*, it was likely that some GOF mutations in this gene were responsible for the acquisition of resistance. Surprisingly, contrary to what might be expected, no mutation was found in the *PDR1* gene in any of the resistant mutants. This observation has led us to hypothesize that there must be other mechanisms of resistance that do not involve *PDR1*. Firstly, we thought that the ABC transporter, *CDR1*, could be regulated by another transcription factor other than the *PDR1* gene, and the observable resistance continued to be the result of the upregulation of an ABC transporter.

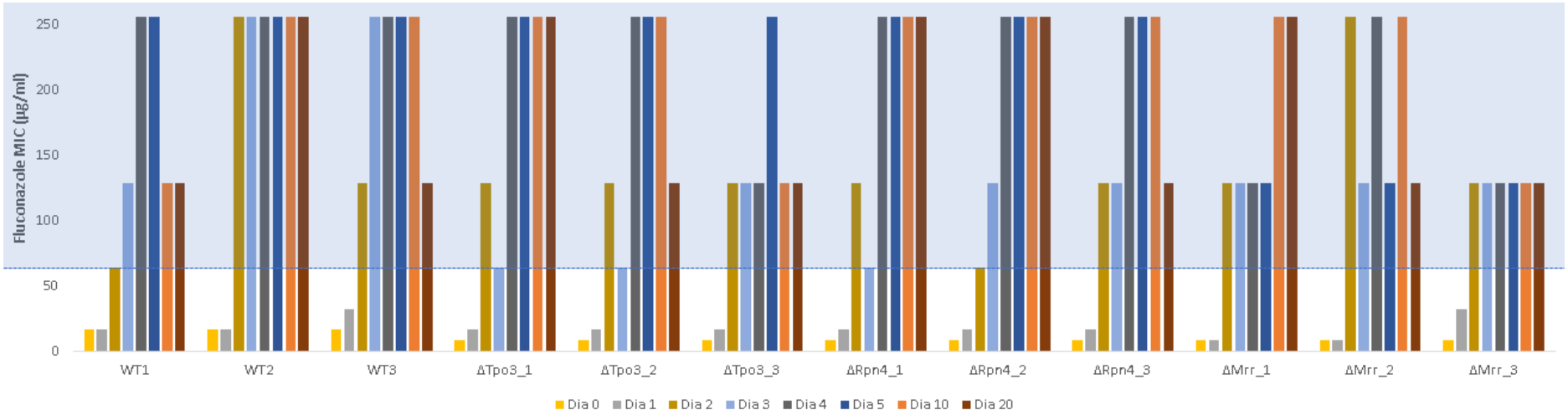


Figure 10: In vitro fluconazole MIC values of *C. glabrata* KChr606, *Δtpo3*, *Δrpn4* and *Δmrr1*strains upon 0, 1, 2, 3, 4, 5, 10 and 20 days of cultivation in the presence of 16µg/ml of fluconazole. Results for triplicate experiments are displayed. The blue area represents the limit at which the strains are resistant to fluconazole.

To test this hypothesis, we determined whether *CDR1* expression was altered in the resistant evolved mutants *Δrpn4* and *Δmrr1* compared to day 0. The results represented in Figure 11 and Figure 12, demonstrated that, no changes in *CDR1* gene expression was found in the evolved *Δrpn4* strains, while only a mild increase in *CDR1* expression was observed in the evolved *Δmrr1* strains. Despite the fact that up to date the upregulation of ABC transporters is considered the main mechanism of resistance to azoles in *C. glabrata*, these results suggest that there are other azole resistance mechanisms, independent of the *PDR1* network, which are not yet described in *C. glabrata*.

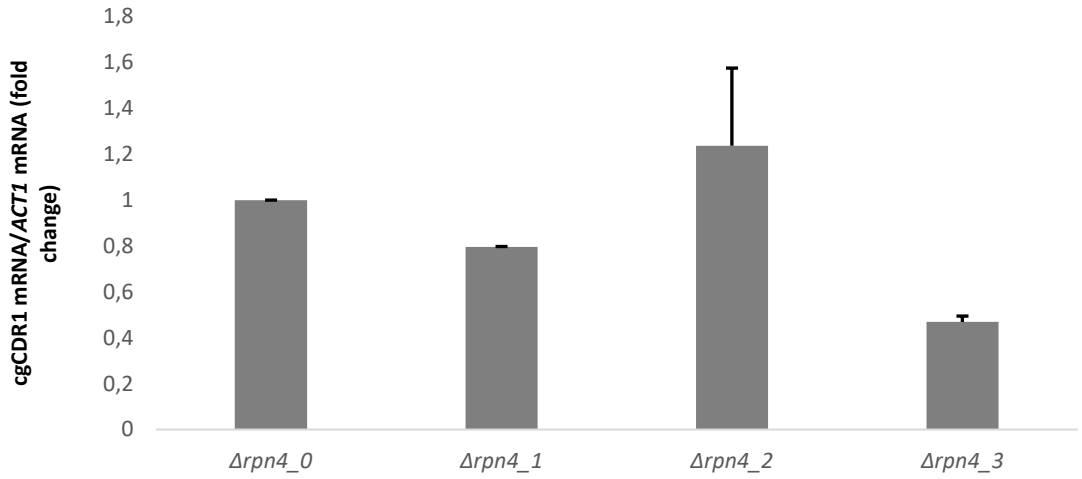


Figure 11: Comparison of the transcript levels of *CgCDR1* gene in the *Δrpn4* (day 0) and the evolved resistant strains *Δrpn4_1*, *Δrpn4_2* and *Δrpn4_3*. The indicated values correspond to the averages obtained by two independent experiments of quantitative real-time PCR. The error bars correspond to the standard deviations. *p<0.05.

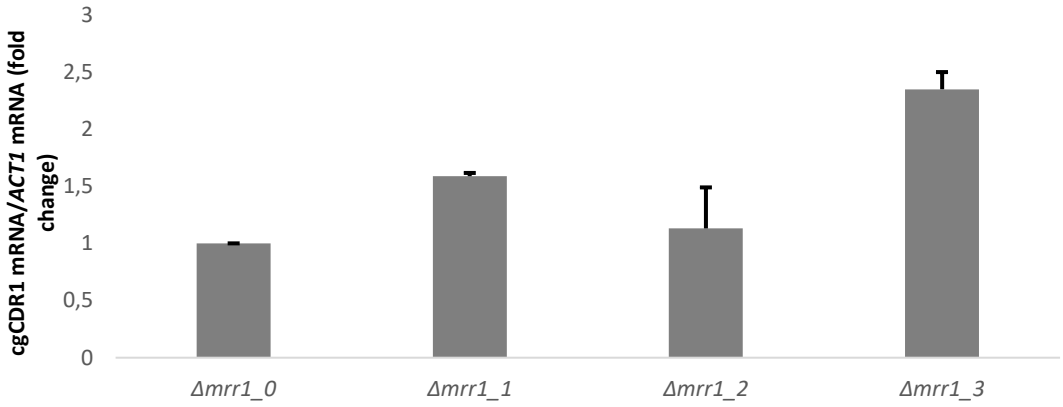


Figure 12: Comparison of the transcript levels of *CgCDR1* gene in the *Δmrr1* (day 0) and the evolved resistant strains *Δmrr1_1, Δmrr1_2* and *Δmrr1_3*. The indicated values correspond to the averages obtained by two independent experiments of quantitative real-time PCR. The error bars correspond to the standard deviations. *p<0.05.

3.2 Fluconazole resistance in clinical isolates is not always dependent on *CgPDR1* GOF mutations

Given the observation that it is possible to evolve strains, in vitro, towards fluconazole resistance, without the up rise of GOF mutations in the *PDR1* gene, it is likely that other factors besides the acquisition of *CgPDR1* GOF mutations must underlie the acquisition of fluconazole resistance in the clinical setting. To test this hypothesis, the incidence of *CgPDR1* mutations were investigated in a collection of *C. glabrata* clinical isolates, harvested from patients attending two major Hospital centers in Portugal (Costa et al., 2016). Among the more than 100 isolates in this collection, the 7 most azole-resistant isolates and 6 most azole-susceptible strains were selected. Upon sequencing the *CgPDR1* gene in these clinical isolates, 13 single amino acid substitutions were registered (Table 7). The aminoacid substitutions in positions 76, 91, 98, 143 e 243 in *CgPDR1* were reported before as polymorphisms found in azole-susceptible and azole-resistant isolates (Ferrari, Ischer et al. 2009), and thus discarded as potential GOF mutations. Substitution in amino acid 33 was only observed in susceptible strains, whereas, 6 of the single amino acid substitutions (positions 282, 376, 453, 753, 847 and 1090) were only identified in azole-resistant isolates (Table 7). It will be interesting to analyze the influence of these mutations in azole resistance, since none of them had been registered before as possible GOF mutations. The mutations in the positions 243 and 391 found only in resistant strains, had already been reported also in azole-susceptible isolates (Ferrari et al., 2009; Tsai et al., 2010). Although most of the isolates present possible GOF mutations in *CgPDR1*, three clinical isolates (corresponding to 37.5% of all azole resistant strains), 50570, 67367 and 73281, do not have any possible GOF mutation. These findings confirm the hypothesis formulated that it is possible to acquire resistance independently of the *CgPDR1*gene, even in the clinical context.

Analyzing the distribution of published GOF mutations in *CgPDR1* (figure 13) it is possible to observe that these are dispersed throughout the whole protein. However, a higher incidence of mutations is registered in areas identified as domains, PID (putative inhibitory domain), FSTFD (fungus-specific transcription factor domain) and AD (putative transcriptional activation domain). In fact, the five mutations identified in clinical isolates, are also in these domains or in nearby regions, following the pattern of GOF mutations already identified. FSTFD is a domain found in several fungal transcription factors, regulating a variety of cellular and metabolic processes, so that mutations in this domain can be expected to translate into a GOF mutation. The same can be expected from the PID. In fact, a GOF mutation, L280F, has already been identified in this domain, leading to azole resistance and increased virulence (Ferrari et al., 2009), Interestingly this mutation was found in a position close to the R282G mutation identified in this study for the first time.

Table 7: Single amino acid substitutions identified in the CgPdr1 of clinical isolates.

Position	33	76	91	98	143	243	282	376	391	453	753	847	1090
Change	S→R	S→P	V→I	L→S	T→P	D→N	R→G	R→L	S→L	D→Y	L→S	V→F	L→S
13		X	X	X	X		G			G			
10774			X			RG			RG				
50570[1]		X	X	X	X								
51800		X	X	X	X							G	
73281[1]		X	X	X	X								
94078			X	X		RG					G		G
65147								G					
67367[1]		X	X	X	X								
MC 273		X	X	X	X								
MC426	X	X	X	X	X								
MC 123		X	X	X	X								
MC 125		X	X	X	X								
MC126	X	X	X	X	X								
MC127		X	X	X	X								

X, identified mutation in both azole-susceptible and azole-resistant isolates; **G**, mutation found only in azole-azole-resistant isolates, possible GOF mutation. **RG**, Reported GOF mutation. In dark-grey azole-resistant isolates. In light-grey azole-susceptible isolates; [1] resistant isolates with no possible GOF mutation.

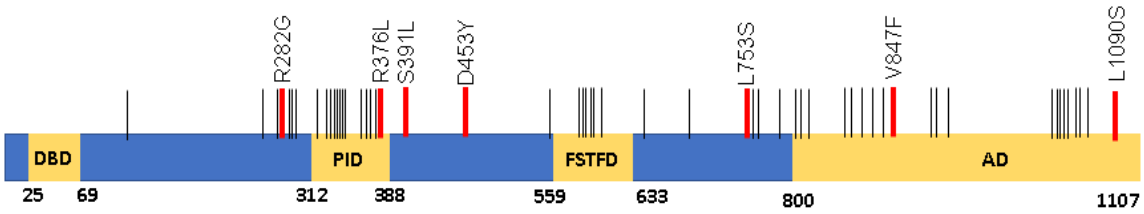


Figure 13: Distribution of *CgPDR1* SNP mutations found in clinical isolates. The domains shown were based on the homology between *S. cerevisiae* Pdr1 and CgPdr1 (Vermitsky and Edlind, 2004). FSTFD, fungus-specific transcription factor domain. PID, putative inhibitory domain. DBD, DNA binding domain. AD, putative transcriptional activation domain. Published GOF mutations are indicated by black bars. The 7 mutations only identified in fluconazole resistant clinical isolates in this study are indicated by red bars.

Under the hypothesis that GOF mutations may arise in other transcription factors involved in fluconazole resistance, besides *CgPDR1*, the sequence of *CgMRR1* and *CgRPN4*, potential genes involved in resistance to azoles (P. Pais, M. Galocha, R. Califórnia, M.C. Teixeira, unpublished results), in the same isolates were also evaluated. The sequences were obtained and compared to the corresponding sequences in the CBS138 reference strain.

In *C. glabrata* the disruption of *CAGL0B03421g*, ortholog of the *MRR1* gene of *C. albicans* (Klimova et al., 2014), was found to lead to a decrease in resistance to azoles. Although *MRR1* gene has not yet been confirmed as responsible for fluconazole resistance in *C. glabrata*, in *C. albicans* it is considered an important azole resistance gene. It has been shown that *CaMRR1* is upregulated in fluconazole resistant *C. albicans* isolates and is considered as the central regulator of the Mdr1 efflux pump in this yeast (Morschhauser et al., 2007). Interestingly, two GOF mutations, P683S and G997V, were reported in *C. albicans* Mrr1 as responsible for constitutive *MDR1* overexpression and multidrug resistance (Morschhauser et al., 2007). In table 8 are represented the mutations found in *CgMRR1* in our collection of clinical isolates. Most of the mutations found were present in both resistant and susceptible isolates. However, 2 mutations at positions 362 and 1290 are only present in the azole resistant clinical isolates 65147 and 10774, respectively. These could be 2 possible GOF mutations. To date, there are no reported GOF mutations for this TF in *C. glabrata*. Two predicted domains were identified in CgMrr1 (figure 14), but none of the 2 possible GOF mutations identified in clinical isolates is located in these regions.

Table 8: Single amino acid substitutions identified in the CgMrr1 of clinical isolates

Position	**115**	**139**	**158**	**274**	**306**	**321**	**362**	**1256**	**1290**	**1294**
Change	**V→I**	**M→V**	**D→Y**	**A→T**	**A→T**	**N→D**	**Y→F**	**P→S**	**D→E**	**L→V**
13						**X**				
10774				**X**	**X**	**X**			**G**	
50570[1]						**X**				
51800						**X**				
73281[1]						**X**		**X**		
94078		**X**		**X**	**X**	**X**				
65147							**G**			
67367[1]						**X**		**X**		
MC 273		**X**	**X**	**X**	**X**	**X**				
MC426						**X**		**X**		
MC 123						**X**				
MC 125	**X**					**X**				
MC126	**X**	**X**				**X**				
MC127	**X**	**X**				**X**				**X**

X, identified mutation in both azole-susceptible and azole-resistant isolates; **G**, mutation found only in azole-azole-resistant isolates. In light-grey azole-susceptible isolates. [1] resistant isolates with no possible GOF mutation in the *PDR1*.

Figure 14. Distribution of *CgMRR1* SNP mutations found in clinical isolates. ZF, Zinc finger domain. FSTFD, fungus-specific transcription factor domain. Domains are based on the data available in the CGD (www.candidagenome.org/). Mutations found only in the resistant clinical isolates analyzed are indicated by red bars.

Rpn4, a transcription factor that stimulates the expression of proteasome genes, was shown to be upregulated in several fluconazole resistant *C. glabrata* clinical isolates (Tsai et al., 2010). Given the possible importance of this gene, eventual mutations in the *RPN4* gene in these clinical isolates was also assessed, aiming at the identification of eventual candidates to GOF mutations underlying azole resistance. For this gene, 4 mutations were found only in azole resistant isolates (Table 9). Curiously the isolate 50570, one of those that did not have any possible GOF mutation in the *PDR1* gene, presents 3 of these mutations, two of which, in adjacent positions, (100 Ins A and 101 Ins Q), corresponding to the insertion of 2 new amino acids in the protein sequence. Given that, it will be interesting to assess if these mutations are indeed responsible, at least partially, for the acquisition of resistance in these clinical isolates.

Table 9: Single amino acid substitutions identified in the CgRpn4 of clinical isolates

Position	86	100	101	160	186	233	334	335	367
Change	P→S	Ins A	Ins Q	V→D	E→K	N→S	D→E	N→T	A→P
13	X			X					
10774		G	G	X	G		X	X	
50570[1]		G	G	X		G	X	X	
51800				X					
73281[1]	X			X					
94078		G	G	X			X	X	
65147									
67367[1]	X			X					
MC273				X			X	X	
MC426	X			X					
MC123				X					X
MC125				X					X
MC126				X					X
MC127				X					X

X, identified mutation in both azole-susceptible and azole-resistant isolates; **G**, mutation found only in azole- azole-resistant isolates. In light-grey azole-susceptible isolates. [1] resistant isolates with no possible GOF mutation in the *PDR1*.

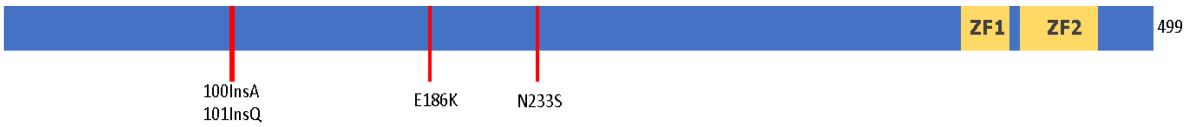


Figure 15: Distribution of *CgRPN4* mutations found in clinical isolates. ZF1 and ZF2, are C2H2-type zinc finger domains. Domains are based on the data available in the CGD (www.candidagenome.org/).. Mutations found only in the resistant clinical isolates analyzed are indicated by red bars.

3.3 Studying the evolution of a *Δpdr1* mutant towards fluconazole resistance: a genome-wide analysis

To test the hypothesis that a *C. glabrata* strain is able to acquire fluconazole resistance even in the absence of the Pdr1 transcription factor, another *Δpdr1* mutant strain available in the lab was use in fluconazole evolution experiments. At this point we were not sure if it was possible to evolve such a susceptible strain towards fluconazole resistance. Prior to fluconazole exposure, the used 66032_ *Δpdr1* strain exhibited a MIC of 4 µg/ml. The experiments were also run in triplicate. It was found that the mutants were able to acquire azole resistance, with a fluconazole MIC of 64 µg/ml, at day 7 (*Δpdr1_1*), at day 8 (*Δpdr1_2*) or at day 2 (*Δpdr1_3*), depending on the strain. The fluconazole MIC value was increased to 128 µg/ml on days 10 and 20. Interestingly, after the acquisition of fluconazole resistance, the evolved strain *Δpdr1_2* exhibited decreased growth rates. The growth curves for the 66032_*Δpdr1* and the evolved *Δpdr1_2* strains, in YDP at 30◦C, were determined (figure 16). It was verified not only a decrease in the doubling time but a decrease in the total biomass concentration reached at stationary phase. While the initial strain has a doubling time of 1.43h, a typical value for *C. glabrata* strains growing in YPD, the mutant showed a considerably higher value, 2.78h. This observation led us to hypothesize that this mutant must have acquired some mutation or mutations that, at the same time as it confers fluconazole resistance, it carries a fitness cost for the mutant. This resistant phenotype may be an advantage in the presence of fluconazole allowing the population to survive but is a disadvantage compared to their fluconazole-susceptible counterparts in a non-stressing environment. This mutant was selected for genomic sequencing and will be referred to in this study as *Δpdr1_2fr* (fluconazole resistant). The other two mutants will also be sequenced soon.

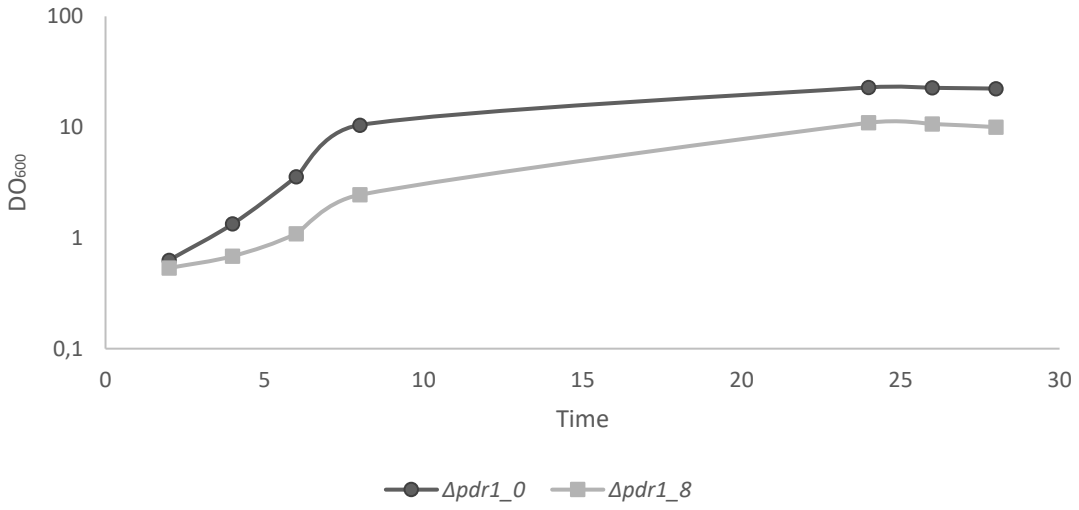


Figure 16: Comparison of growth curves at day 0 of *C. glabrata* 66032_*Δpdr1* and at day 8 of *Δpdr1_2fr* cell populations, in liquid YPD medium at 30◦C, in the absence of fluconazole. Measured in terms of variation in OD_{600}. The displayed growth curves are representative of the two independent experiments.

In order to understand the genetic changes underlying the increased fluconazole resistance acquired by the *Δpdr1_2fr* evolved strain, when compared with the fluconazole susceptible *Δpdr1* strain, the genome sequences of both strains were obtained. The sequencing data was submitted to the GenomeScope software to analyze k-mer distribution and estimate preliminary genome features of the strains under study. Using k-mer size = 21, at an average k-mer coverage of 187X for the susceptible strain and 162X for the resistant strain, the estimated haploid genome size was around 11.7 Mbp for both strains (Table 10), which is very similar to the reference genome size of 12.3 Mbp. These results are consistent with the notion that *C. glabrata* has an haploid genome. Moreover, the analysis revealed a variety of only 0.28-0.30% across the entire genome, indicating that the genome of these strains has very few divergent sequences, resulting in scarce heterozygous properties. GenomeScope also estimated a reduced read error rate (0.05%), indicating that read preprocessing and correction was effective (Table 10).

Table 10: GenomeScope metrics for fluconazole susceptible 66032__*Δpdr1* and *Δpdr1_2fr* strains genome features.

Strain	**Fluconazole-susceptible**		**Fluconazole-resistant**	
k-mer = 21	**k-mer coverage = 187x**		**k-mer coverage = 162x**	
Property	**minimun**	**maximum**	**minimun**	**maximun**
Heterozygosity (%)	0.302329	0.305718	0.286074	0.289718
Genome Haploid Length (bp)	11,739,732	11,746,341	11,755,549	11,762,340
Genome Repeat Length (bp)	475,379	475,646	504,221	504,512
Genome Unique Length (bp)	11,264,353	11,270,695	11,251,328	11,257,828
Model Fit (%)	97.3289	97.8504	96.9997	97.4619
Read Error Rate (%)	0.0520156	0.0520156	0.0527271	0.0527271

To identify variants occurring in the fluconazole resistant strain that could be associated with the correspondent resistance phenotype, high-quality reads from both fluconazole susceptible and resistant strains were aligned against the *C. glabrata* reference genome with approximately 792X coverage. Notably, 99.3% of the reads from each strain were mapped to the reference genome, indicating a low level of read contamination. Variant calling was performed using two different tools, BCFTools and GATK, considered the two best tools available (Hwang et al., 2015; Sandmann et al., 2017). Since different results were obtained by the two tools (Table 11), the sum of the variants identified by each of them was considered. The most promising alterations will be confirmed by sanger sequencing before continuing to further studies.

Table 11: Results obtained for variant calling performed with two different tools, BCFTools and GATK.

		BCFTOOLS	GATK
	Susceptible Strain Variants	60297	66515
	Resistant Strain Variants	60234	66425
Resistant	Total	372	305
Strain	Coding region	64	112
Specific	Coding region non-synonymous	28	38
Variants	Indels	7	12

A total of 64 possible non-synonymous mutations in protein coding regions were identified in the *Δpdr1_2fr* mutant, when compared to the *Δpdr1* strain (Table 12). Of these, 28 and 38 non-synonymous mutations were identified through BCFTools and GATK, respectively. Only two of them were identified by the 2 tools in the same position, in ORFs *CAGL0M12617g* (949G>T) and *CAGL0H01144g* (1196G>A). Since mutations in coding regions are more propitious to be

responsible for some phenotypic alteration the focus of this work was directed to its analysis. The analysis of the distribution of protein coding mutation only present in *Δpdr1_2fr* per chromosome (figure 17) reveals that they are unevenly distributed between the chromosomes. Chromosomes L and J have a higher incidence of mutations than the remaining, contrariwise some chromosomes do not have any mutation, D and A.

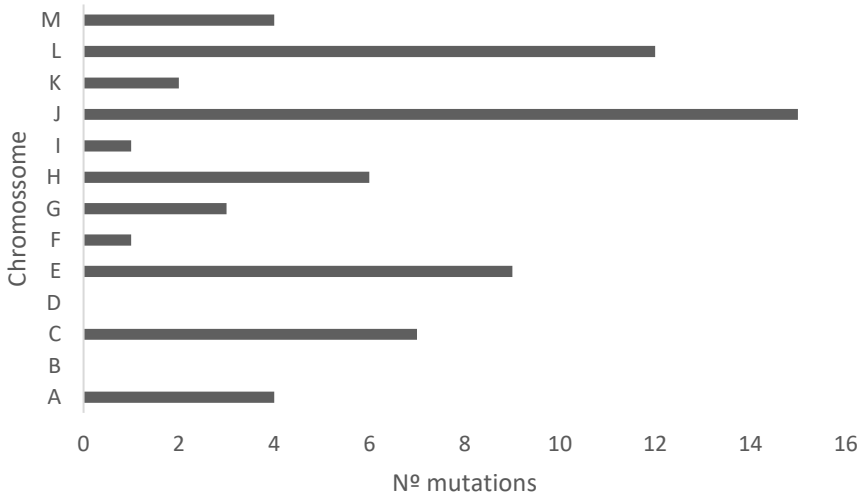


Figure 17: Number of mutations in coding regions only present in *Δpdr1_2fr* per chromosome.

Table 12: List of mutations in coding regions only present in the *Δpdr1_2fr* mutant.

Chromossome	Position	Position in Gene and Variant	Effect	Predicted Impact	*ORF C. glabrata*	Gene *C. glabrata*	*S. cerevisiae ortholog*
A	134918	c.3557A>G	missense_variant	Moderate	*CAGL0A01284g*	*EPA10*	-
A	135227	c.3866G>A	missense_variant	Moderate	*CAGL0A01284g*	*EPA10*	-
A	146594	c.1207C>A	missense_variant	Moderate	*CAGL0A01408g*	*CAGL0A01408g*	*PGD1*
A	245412	c.730G>T	missense_variant	Moderate	*CAGL0A02255g*	*CAGL0A02255g*	-
C	11924	c.1016C>T	missense_variant	Moderate	*CAGL0C00209g*	*AWP7*	-
C	98969	c.2056A>G	missense_variant	Moderate	*CAGL0C00968g*	*CAGL0C00968g*	-
C	99114	c.2201G>C	missense_variant	Moderate	*CAGL0C00968g*	*CAGL0C00968g*	-

C	192806	c.10-330C>T	intron_variant	Modifier	*CAGL0C01837g*	*RER1*	-
C	192826	c.10-350C>T	intron_variant	Modifier	*CAGL0C01837g*	*RER1*	-
C	194981	c.199-167delC	intron_variant	Modifier	*CAGL0C01859g*	*CAGL0C01859g*	-
C	194984	c.199-169A>C	intron_variant	Modifier	*CAGL0C01859g*	*CAGL0C01859g*	-
E	12553	c.675T>G	missense_variant	Moderate	*CAGL0E00187g*	*CAGL0E00187g*	-
E	15735	c.5312A>G	missense_variant	Moderate	*CAGL0E00231g*	*CAGL0E00231g*	-
E	15738	c.5309T>A	missense_variant	Moderate	*CAGL0E00231g*	*CAGL0E00231g*	-
E	15742	c.5305C>G	missense_variant	Moderate	*CAGL0E00231g*	*CAGL0E00231g*	-
E	12759	c.469G>A	missense_variant	Moderate	*CAGL0E00187g*	*CAGL0E00187g*	-
E	12809	c.419C>T	missense_variant	Moderate	*CAGL0E00187g*	*CAGL0E00187g*	-
E	16472	c.4575G>T	missense_variant	Moderate	*CAGL0E00231g*	*CAGL0E00231g*	-
E	673125	c.1567_1568insGACCCAAAACCTGAAGACCCCTCACACA	frameshift_variant&stop_gained	High	*CAGL0E06666g*	*EPA2*	-
E	682309	c.1022_1023insTTCAGTTAACCCATCTTCTGTGAACCCATCTTCAGTTAACCCATCCTCAGTCAACCCATCTTCCGTCAACCCTTCAAGCAAGCCAGTTGATCCTTCTCCAGCTGACCCATCACACAACCCATCTTCCGTTAACCCATCTTCTGTGAACCCATCTTCCGTCAACCCATC	disruptive_inframe_insertion	Moderate	*CAGL0E06688g*	*EPA3*	-
F	461730	c.938G>A	missense_variant	Moderate	*CAGL0F04587g*	*CAGL0F04587g*	*PSY4*
G	86970	c.1158_1159insCCTCCTATGGCTTCAGCA	conservative_inframe_insertion	Moderate	*CAGL0G00968g*	*CAGL0G00968g*	*VRP1*
G	86976	c.1153_1154insCTATGGCTT	conservative_inframe_insertion	Moderate	*CAGL0G00968g*	*CAGL0G00968g*	*VRP1*

G	395265	c.1387A>T	missense_variant	Moderate	*CAGL0G04125g*	*CAGL0G04125g*	*SAG1*
H	2621	c.1091T>G	stop_gained	High	*CAGL0H00110g*	*CAGL0H00110g*	
H	105235	c.1196G>A	missense_variant	Moderate	*CAGL0H01144g*	*CAGL0H01144g*	*ZIP1*
H	582566	c.469_474dupAACATG	conservative_inframe_insertion	Moderate	*CAGL0H05929g*	*CAGL0H05929g*	*SYH1*
H	938138	c.280T>A	missense_variant	Moderate	*CAGL0H09592g*	*CAGL0H09592g*	*TIR1*
H	1045612	c.1670T>C	missense_variant	Moderate	*CAGL0H10626g*	*AWP13*	-
H	1047745	c.3803G>A	missense_variant	Moderate	*CAGL0H10626g*	*AWP13*	-
I	960373	c.3701_3702insTAGCATGTCCTCATCTTCTTCATCTAGCATGTCCTCATCTTCTTCATCCAGCATGTCCTCATCTTCTTCATC	disruptive_inframe_insertion	Moderate	*CAGL0I10098g*	*PWP7*	-
J	166571	c.5469_5486delTGAAGGCGGTTCTGGTTC	conservative_inframe_deletion	Moderate	*CAGL0J01774g*	*CAGL0J01774g*	-
J	166933	c.5129_5140delAAGGTGGCTCTG	disruptive_inframe_deletion	Moderate	*CAGL0J01774g*	*CAGL0J01774g*	-
J	167013	c.5060_5061insCTC	disruptive_inframe_insertion	Moderate	*CAGL0J01774g*	*CAGL0J01774g*	-
J	168287	c.3787T>C	missense_variant	Moderate	*CAGL0J01774g*	*CAGL0J01774g*	-
J	168290	c.3784G>A	missense_variant	Moderate	*CAGL0J01774g*	*CAGL0J01774g*	-
J	169242	c.2817_2818insGGCTCT	conservative_inframe_insertion	Moderate	*CAGL0J01774g*	*CAGL0J01774g*	-
J	175946	c.1171_1206dupCCTCCTGCAAACGCACCTGCTAATCCTCCTGCTAAT	conservative_inframe_insertion	Moderate	*CAGL0J01800g*	*CAGL0J01800g*	-
J	176036	c.1117C>G	missense_variant	Moderate	*CAGL0J01800g*	*CAGL0J01800g*	-
J	176054	c.1098_1099insTATTTTTGTATCTAATTTCTA	stop_gained&conservative_	High	*CAGL0J01800g*	*CAGL0J01800g*	-

		GCACTTACTTAAGTTTTGAAAAACCGCAGGAGTTATCATGAAAGA	inframe_insertion				
J	251506	c.1387_1388insTGCCCTCATCTTCAGTTGAGCCATCCTCATCAGTTGAGCCATCCTCATCAGTGG	conservative_inframe_insertion	Moderate	*CAGL0J02530g*	*CAGL0J02530g*	-
J	251717	c.1176_1177insA	frameshift_variant	High	*CAGL0J02530g*	*CAGL0J02530g*	-
J	251720	c.1173delT	frameshift_variant	High	*CAGL0J02530g*	*CAGL0J02530g*	-
J	251838	c.1055_1056insATCAGTTGATACCTCTTC	disruptive_inframe_insertion	Moderate	*CAGL0J02530g*	*CAGL0J02530g*	-
J	1163951	c.1138_1149delCCATCTTCAATG	conservative_inframe_deletion	Moderate	*CAGL0J11891g*	*AWP3*	-
J	1175030	c.2127_2174dupTCCAAGTCCAAGTCCAAGTCCAAGTCCAAGTCCAAGTCCAAGTCCAAG	disruptive_inframe_insertion	Moderate	*CAGL0J11968g*	*EPA15*	-
K	766878	c.1936_1989delATAGTTGAGAGGGTAGTTGAGGATGAGGTAGTTGAGAGGGTAGTTGAGGATGAG	conservative_inframe_deletion	Moderate	*CAGL0K07700g*	*CAGL0K07700g*	*BUD27*
K	1299074	c.1300C>T	missense_variant	Moderate	*CAGL0K13024g*	*AED1*	-
L	1632	c.4300G>A	missense_variant	Moderate	*CAGL0L00157g*	*CAGL0L00157g*	-
L	1727	c.4205C>T	missense_variant	Moderate	*CAGL0L00157g*	*CAGL0L00157g*	-
L	1729	c.4203C>A	stop_gained	High	*CAGL0L00157g*	*CAGL0L00157g*	-
L	1794	c.4138G>A	missense_variant	Moderate	*CAGL0L00157g*	*CAGL0L00157g*	-
L	1826	c.4106C>T	missense_variant	Moderate	*CAGL0L00157g*	*CAGL0L00157g*	-
L	1899	c.4033A>G	missense_variant	Moderate	*CAGL0L00157g*	*CAGL0L00157g*	-
L	1904	c.4028C>T	missense_variant	Moderate	*CAGL0L00157g*	*CAGL0L00157g*	-
L	18493	c.9044T>C	missense_variant	Moderate	*CAGL0L00227g*	*CAGL0L00227g*	-

L	22743	c.4774_4788delGGTGGCTCTGGCTCT	conservative_inframe_deletion	Moderate	*CAGL0L00227g*	*CAGL0L00227g*	-
L	24806	c.2716_2730delAACCCAGGTGGCTCT	conservative_inframe_deletion	Moderate	*CAGL0L00227g*	*CAGL0L00227g*	-
L	1068288	c.3947T>A	missense_variant	Moderate	*CAGL0L09911g*	*CAGL0L09911g*	-
L	1429552	c.2859G>C	missense_variant	Moderate	*CAGL0L13299g*	*EPA11*	-
M	13016	c.1389A>G	missense_variant	Moderate	*CAGL0M00132g*	*EPA12*	-
M	848485	c.397G>A	missense_variant	Moderate	*CAGL0M08492g*	*PIR3*	*PIR1*
M	1250742	c.949G>T	stop_gained	High	*CAGL0M12617g*	*CAGL0M12617g*	*YRB2*
M	1396019	c.472C>T	missense_variant	Moderate	*CAGL0M14091g*	*CAGL0M14091g*	-

3.3.1 Searching for mutations that may underlie the fluconazole resistance phenotype

In total, 64 mutations were identified in the evolved strain, when compared to the parental strain, affecting 34 different genes (Table 13). Among these mutations, two possible scenarios could be considered, the modification of a single gene was enough for the induction of resistance, or mutations in a set of genes originated the acquisition of resistance.

Table 13: Description of genes with mutations found only in the genome sequencing of *Δpdr1_2fr*.

Group Function	*C. glabrata* designation	*S. cerevisiae* homolog	Description
RNA processing	*CAGL0M12617g*	*YRB2*	Ortholog(s) have role in regulation of chromatin silencing at telomere, ribosomal small subunit export from nucleus and cytosol
	CAGL0K07700g	*BUD27*	Ortholog(s) have role in RNA polymerase I assembly, RNA polymerase II core complex assembly, RNA polymerase III assembly a complex
	CAGL0H05929g	*SYH1*	Ortholog(s) have role in nuclear pore distribution and P-body

	CAGL0A01408g	*PGD1*	Ortholog(s) have RNA polymerase II activating transcription factor binding
DNA damage response	*CAGL0F04587g*	*PSY4*	Ortholog(s) have protein phosphatase regulator activity, role in negative regulation of DNA damage checkpoint
Cytoskeleton organization	*CAGL0G00968g*	*VRP1*	Ortholog(s) have actin binding activity
Response to Stress	*CAGL0H09592g*	*TIR1*	Putative GPI-linked cell wall protein, response to stress
	CAGL0C00209g/AWP7	No similarity	Putative adhesin-like cell wall protein; belongs to adhesin cluster IV; predicted GPI-anchor
Cell adhesion	*CAGL0I10098g/PWP7*	No similarity	Cell wall protein that mediates adhesion to endothelial cells; predicted GPI anchor and signal peptide; belongs to adhesin cluster II
	CAGL0C00968g	No similarity	Adhesin-like protein with a predicted role in cell adhesion; belongs to adhesin cluster VII; predicted GPI-anchor
	CAGL0H10626g/AWP13	No similarity	Predicted cell wall adhesin with a role in adhesion; belongs to adhesin cluster III; predicted GPI anchor; contains tandem repeats
	CAGL0K13024g/AED1	No similarity	Adhesin-like protein required for adherence to endothelial cells; belongs to adhesin cluster III; predicted GPI anchor; 6 tandem repeats
	CAGL0L13299g/EPA11	No similarity	Putative adhesin; belongs to adhesin cluster I
	CAGL0G04125g	*SAG1*	Protein with similarity to S. cerevisiae Sag1 agglutinin, involved in cell adhesion; predicted GPI-anchor
Cell Cycle	*CAGL0H01144g*	*ZIP1*	Ortholog(s) have SUMO polymer binding activity
Other metabolisms	*CAGL0M14091g*	No similarity	Putative quinone reductase/NADPH dehydrogenase; gene is upregulated in azole-resistant strain

Cell wall organization	*CAGL0M08492g /PIR3*	*PIR1*	Pir protein family member, putative cell wall component
Unknown function	*CAGL0H00110g*	No similarity	Adhesin-like protein with internal repeats; predicted GPI-anchor; likely a C-terminal fragment of a single ORF with *CAGL0H00132g*; belongs to adhesin cluster V
	CAGL0L00227g	No similarity	Putative adhesin with glycine and serine rich repeats; belongs to adhesin cluster V
	CAGL0M00132g/EPA12	No similarity	Putative adhesin-like cell wall protein; belongs to adhesin cluster I
	CAGL0E06666g/EPA2	No similarity	Epithelial adhesion protein; predicted GPI-anchor; belongs to adhesin cluster I
	CAGL0E06688g/EPA3	No similarity	Epithelial adhesion protein; belongs to adhesin cluster I; GPI-anchored
	CAGL0E00231g	No similarity	Putative adhesin-like protein; contains tandem repeats and a predicted GPI-anchor; belongs to adhesin cluster III
	CAGL0J11968g/EPA15	No similarity	Putative adhesin-like cell wall protein; belongs to adhesin cluster I
	CAGL0L09911g	No similarity	Putative adhesin-like cell wall protein; 5 tandem repeats; predicted GPI-anchor
	CAGL0E00187g	No similarity	Putative adhesin-like protein; belongs to adhesin cluster IV
	CAGL0J01774g	No similarity	Putative adhesin-like protein; has glycine and serine rich repeats; belongs to adhesin cluster VI
	CAGL0L00157g	No similarity	Putative adhesin-like protein; multiple tandem repeats; predicted GPI-anchor; belongs to adhesin cluster III
	CAGL0J02530g	No similarity	Putative adhesion protein; predicted GPI-anchor; belongs to adhesin cluster VI
	CAGL0A01284g/EPA10	No similarity	Putative adhesin-like protein; belongs to adhesin cluster I

CAGL0J01800g	No similarity	Putative adhesin-like protein; belongs to adhesin cluster VI
CAGL0J11891g/AWP3	No similarity	Putative adhesin-like protein; identified in cell wall extracts by mass spectrometry; belongs to adhesin cluster VI
CAGL0C01859g	No similarity	Has domain(s) with predicted integral component of membrane localization
CAGL0A02255g	No similarity	Protein of unknown function

[a] Descriptions are based on those in the CGD (www.candidagenome.org/).

Regarding the functional categories that are mostly present within the mutated genes, the group of adhesins stands out. A total of 21 of the 34 mutated genes are described as adhesins or adhesin-like encoding genes (Table 13), although much of them lack functional characterization. 8 of them are described as having a function involved in cell adhesion. This group of genes are commonly associated with virulence and biofilm formation. As such, it would be interesting to determine if there was any change in biofilm formation capacity in the evolved strain, when compared to the parental strain. Since cells growing in biofilms exhibit increased drug resistance, is seems reasonable to hypothesize that mutations in various adhesins may change the cell ability to grow in biofilms, to form cell aggregates and to develop drug resistance. As observed in figure 18, adhesins belonging to clusters I, III and VI are the most mutated in the fluconazole resistant strain. Interestingly, recent studies are beginning to suggest that there is an interplay between azole drug resistance and adhesion. Indeed, it was shown that adherence is positively regulated by the Pdr1 transcription factor, through the upregulation of the adhesin encoding gene *EPA1* (adhesin cluster I) (Vale-Silva et al., 2016). Interestingly, in an unpublished study from our group, the Epa3 adhesin was shown to confer azole drug resistance, affecting the intracellular accumulation of the drug (Cavalheiro et al., unpublished results).

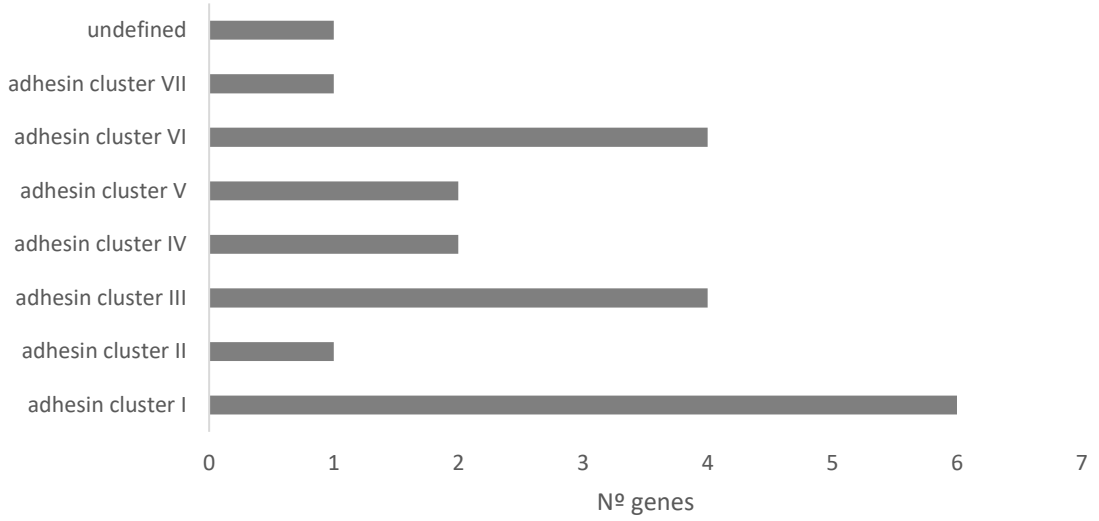


Figure 18: Distribution of the genes described as adhesins-like coding by the different adhesins-like clusters.

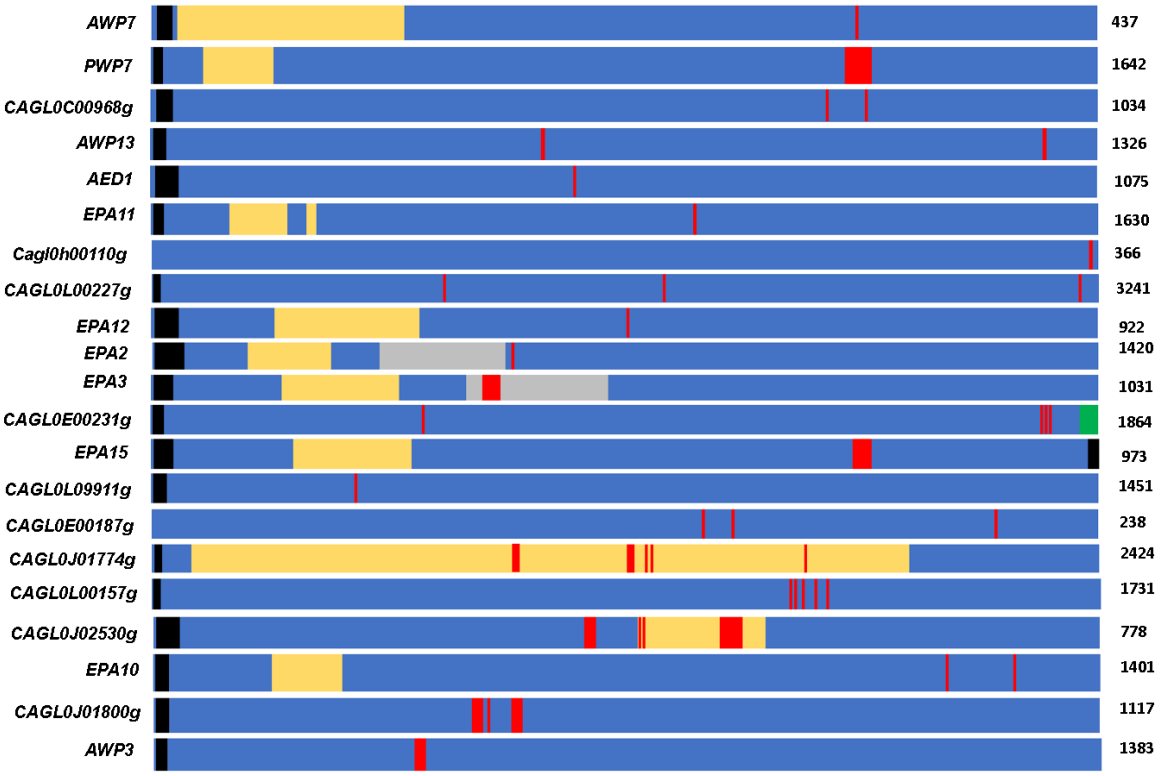


Figure 19: Distribution of the predicted mutations in the adhesins-like genes. Black bars: signal peptide. Yellow region: described domains. Red bars: identified mutations. Green bars: transmembrane domains. Grey region: proline rich extension region. [a] Domains and motifs are based on the data available in the CGD (www.candidagenome.org/).

Analyzing the distribution of the observed mutations in adhesins-like genes, it is possible to observe that 3 of the mutations affect described domains. Interestingly, the Epa3 adhesin has an insertion in a described proline rich region. These proline rich regions, seem to be related to adherence to tissues (Nicholson et al., 1986; Perfect et al., 1998). Given the apparent relationship of this gene with fluconazole resistance, discovered by our group, it will be interesting to assess the impact of the observed mutation found in azole resistance. Several mutations are located in a described domain of the gene *CAGL0J01774g*, however, nothing is known about this domain. Given the large number of mutations in this domain, it is expected to have some influence on the activity of the encoded protein, thereby this adhesin may be a good candidate to analyze in the context of azole resistance. Lastly, 4 predicted mutations were found in a domain of ORF *CAGL0J02530g*, this domain being described as a hexapeptide repeat region. These regions are characteristic of transferases, class of enzymes that mediate the transfer of specific functional groups between molecules. Some transferases have already been related to antifungal drug resistance, namely flucytosine, in some *Candida* species (Kanafani and Perfect, 2008). The last mutation on this gene is a frameshift variant which truncate 48.1% of the protein, whereby, its function is most likely lost. These 3 adhesins with predicted mutations seem to be of particular interest to be studied, however, many of the remaining adhesins identified here still lack functional characterization, therefore, a possible role in the observed fluconazole resistance phenotype should not be discarded.

Considering the possibility that a modification of a single gene may be responsible for the acquisition of azole resistance in the *Δpdr1_2fr* strain, an initial analysis of all mutated genes was made, considering their described functions, observable phenotypes, and type of mutations that they suffered. Eight of them were selected as potential candidates to be involved in resistance to azoles (Table 14) and are analyzed in greater detail below. It is interesting to point out that besides fluconazole resistance, the evolved strain was also found to display decreased exponential growth rate and to reach a lower total final biomass. Therefore, genes with a previously hinted influence in these two phenotypes would be great candidates to be responsible for the acquired phenotypes.

Four of the selected genes have functions related to RNA processing, among which three appear to be associated with resistance to chemical compounds (table 15). The disruption of ORFs *CAGL0K07700g* and *CAGL0A01408g* seems to cause a decrease in the resistance to a wide variety of chemical compounds, including some antifungals. Hence, it will be relevant to study them in greater detail. Even more interesting are the ORFs *CAGL0F04587g* and *CAGL0G00968g* that, in addition to appearing to be related to resistance to a wide variety of compounds and decreased resistance to oxidative stress (table 16), are known to confer fluconazole resistance. Additionally, there is also great interest in analyzing ORFs *CAGL0M14091g*, *CAGL0H09592g* and

CAGL0M08492g in detail. The first, because it was reported to be upregulated in an azole-resistant strain, the second since it has a function related to stress response (table 16), and the third for being involved in cell wall organization and its ortholog shown to be fluconazole induced in *C. albicans*.

Table 14: List of genes selected for its potential to be involved in the observable phenotype.

***C. glabrata* designation**	***S. cerevisiae* homologue**	**Designation adopted**	**Selective characteristic**
CAGL0K07700g	*BUD27*	*CgBUD27*	Disruption in orthologs causes decreased growth rate; Involved in resistance to several chemical compounds
CAGL0A01408g	*PGD1*	*CgPDG1*	Disruption in orthologs causes decreased resistance to several chemical compound
CAGL0F04587g	*PSY4*	*CgPSY4*	Disruption in orthologs causes decreased fluconazole resistance
CAGL0G00968g	*VRP1*	*CgVRP1*	Disruption in orthologues causes decreased growth rate; and decreased fluconazole resistance
CAGL0M08492g /PIR3	*PIR1*	*CgPIR3*	Cell wall organization function; fluconazole induced orthologue
CAGL0M14091g	No similarity	*CAGL0M14091g*	Upregulated in azole-resistant strain
CAGL0H09592g	No similarity	*CAGL0H09592g*	Involved in stress response
CAGL0M12617g	*YRB2*	*CgYRB2*	Stop codon mutation in a described domain

Table 15: Genes with mutations identified only in *Δpdr1_2fr* whose disruption in *C. glabrata* or *S. cerevisiae* orthologue causes alteration in resistance to various chemical compounds. Phenotypes are based on those in the SGD (www.yeastgenome.org).

GROUP	CHEMICAL COMPOUND	NULL MUTANT EFFECT ON RESISTANCE			
		CAGL0K07700g/BUD27	*CAGL0A01408g/PGD1*	*CAGL0F04587g/PSY4*	*CAGL0G00968g/VRP1*
ANTIFUNGAL DRUG	sirolimus	Decreased	Increased	Increased	Decreased
	cycloheximide	Decreased	Decreased	Increased	Decreased
	miconazole	Decreased	-	-	-
	fenpropimorph	Increased	-	Decreased	Decreased
	Fluconazole	-	-	Decreased	Decreased
	flucytosine	-	-	-	Decreased
	caspofungin	-	-	-	Increased
	streptomycin	-	-	-	-
ANTIFUNGAL AGENT	CTBT	Decreased	Decreased	-	-
	sodium arsenite	Decreased	-	-	-
	ascomycin	-	-	Decreased	-
	phleomycin D1	-	-	Decreased	-
	benzethonium chloride	-	-	Decreased	-
	monensin A	-	-	-	Decreased
	concanamycin A	-	-	-	Decreased
ANTIMICROBIAL AGENT	hygromycin B	Decreased	-	-	Decreased
	Ethanol	Decreased	Decreased	-	Decreased
	paromomycin	Decreased	-	-	-
	cordycepin	Decreased	Decreased	-	Decreased
	bleomycin	Increased	-	Decreased	Decreased
	farnesol	-	Decreased	-	-
	tunicamycin	-	Decreased	-	Decreased

doxorubicin	-	Decreased	-	-
tirapazamine	-	Decreased	-	-
propolis extract	-	Decreased	-	Decreased
idarubicin	-	-	Decreased	-
sulfanilamide	-	-	-	Decreased
mycophenolic acid	-	-	-	Decreased
valinomycin	-	-	-	Decreased

Table 16: Genes with mutations identified only in *Δpdr1_2fr* whose disruption causes alteration in stress response.

***C. glabrata* DESIGNATION**	***S. cerevisiae* HOMOLOG**	**NULL MUTANT EFFECT ON STRESS RESISTANCE**	**TYPE OF STRESS**
CAGL0K07700G	*BUD27*	Increased	zinc deficiency
CAGL0A01408G	*PGD1*	Decreased	oxidative stress
CAGL0F04587G	*PSY4*	Decreased	oxidative stress; zinc deficiency
CAGL0G00968G	*VRP1*	Decreased	hyperosmotic; oxidative stress
CAGL0H09592G	*TIR1*	Decreased	oxidative stress

To predict the exact effect on the protein activity of the identified mutations, it would be necessary for the protein to be very well described, and even then, it is still in most cases impossible to draw any definite conclusions about the impact of these modifications. However, if the mutation confers a premature stop codon, it appears safe to assume that, in most cases, it will lead to protein inactivation. Frameshift mutations are another case in which an inactivation of the protein will be predicted. In these two types of mutation the inactivation will always depend on the region where the protein is prematurely truncated. In Table 17 are listed the stop codon and frameshift mutations found only in the genome sequencing of *Δpdr1_2fr* evolved mutant. In four of the mutations more than 40% of the protein sequence is truncated, whereby, it is quite likely that they should cause an inactivation of the proteins. *CAGL0E06666g* encodes an epithelial adhesion protein; *CAGL0J01800g* encodes a putative adhesin-like protein and *CAGL0L00157g* encodes an adhesin-like protein. Although these three genes appear to be related to cell adhesion, its function is unknown. Thus, they may have a possible impact, but little can be inferred about its influence on resistance to fluconazole. The ORF *CAGL0J02530g* encodes a putative adhesion

protein, and its possible impact has been analyzed previously in this section. In the other two mutations, less than 3% of the protein sequence is truncated, so these mutations may not have any impact in the functionality of the protein. It would be interesting to determine if the disruption of these genes in the *Δpdr1* mutant at day 0 would have any impact on fluconazole resistance. Given the known function of *CAGL0M12617g* in RNA processing events and the fact that the stop codon affects a described Seripauperin and TIP1 family domain, this gene will be part of the set of genes whose mutations may have an impact on fluconazole resistance and will be discussed in more detail below.

Table 17: List of stop codon and frameshift mutations found only in the genome sequencing of *Δpdr1_2fr*.

C. glabrata **GENE**	***S. cerevisiae*** **HOMOLOG**	**MUTATION EFFECT**	**DESCRIPTION**	**% OF TRUNCATED PROTEIN**
CAGL0J02530G	No similarity	Frameshift variant	Putative adhesion protein	48.1
CAGL0E06666G	*EPA2*	Frameshift variant	Epithelial adhesion protein	63.2
CAGL0H00110G	No similarity	Stop gained	Adhesin-like protein	0.5
CAGL0J01800G	No similarity	Stop gained	Putative adhesin-like protein	66.2
CAGL0L00157G	No similarity	Stop gained	Putative adhesin-like protein	76.8
CAGL0M12617G	*YRB2*	Stop gained	RNA processing function	2.5

3.3.2 Analyzing candidates

3.3.2.1 Candidate genes whose activity is likely to cause growth defects

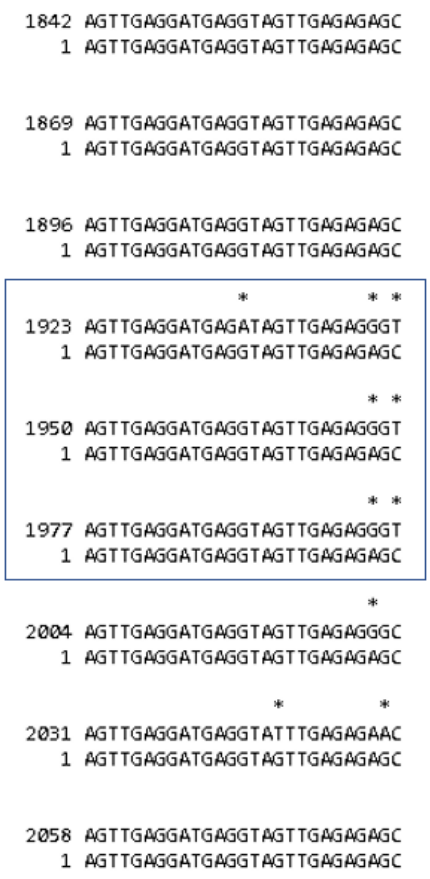
3.3.2.1.1 *CAGL0K07700g/BUD27*

CgBUD27 (ORF *CAGL0K07700g*) has 14 orthologous genes in *Candida* species, and an ortholog in *S. cerevisiae*, *BUD27*. Interestingly, the deletion of the *S. cerevisiae* orthologue of *CgBUD27* is known to lead to decreased exponential growth rate (Deplazes et al., 2009; Li et al., 2009). Since this growth deficiency is one of the phenotypes displayed by the evolved *Δpdr1_2fr* strain, it is possible to hypothesize that the observed mutation in the *CgBUD27* sequence may contribute to this phenotype. Bud27 is described as an unconventional prefoldin protein involved in translation initiation, required for correct assembly of RNAP I, II, and III in an Rpb5-dependent manner. *Δbud27* mutants have inappropriate expression of nutrient sensitive genes due to

translational derepression of the Gcn4 transcription factor and diploid mutants show random budding. For the sake of simplicity, *CAGL0K07700g* will be here referred as *CgBUD27*, despite not having an official standard name. A deletion of 51 nucleotides was found in the sequence of *CgBUD27* in the *Δpdr1_2fr* evolved resistant mutant (figure 20). This deletion resulted in a mutated protein, 17 amino acids removed (figure 21), which is likely to cause some impact in its functionality.

Figure 20: Distribution of Cg*BUD27* specific mutations found in the evolved resistant mutant *Δpdr1_2fr*. Mutations are indicated by red bars. DUF: Domain of unknown function. PFD: Prefoldin subunit. Domains are based on the data available in the CGD (www.candidagenome.org/).

Figure 21: Representation of the deletion found in the gene Cg*BUD27* of the *ΔPdr1_2fr* evolved resistant mutant. Upper sequence corresponds to reference *C. glabrata* CBS138, and Lower sequence corresponds to *Δpdr1_2fr*.



The presence of simple minisatellites (DNA tandem repeats) in *CgBUD27* was reported (Thierry et al., 2008). In *S. cerevisiae*, this type of repeated sequences are encountered in genes encoding cell wall proteins (Richard and Dujon, 2006). Using Tandem Repeats Finder Program (https://tandem.bu.edu/trf/trf.html), was determined where these sequences are located (figure 22). The identified deletion (1936deletion1989) affects 3 of these replicates, in such a way that it eliminates large part of these tandem repeats. However, in *CgBUD27*, given its function, these sequences are not expected to be related to cell wall proteins.

Figure 22: tandem repeats found in *CgBUD27*. Blue box highlights the repetition in which the deletion occurs. * represent the variations in the repeated pattern. Data obtained using the "Tandem Repeats Finder Program" (Benson, 1999).

ScBUD27 is required for efficient translation initiation in *S. cerevisiae*. Yeast cells use budding as asexual reproduction by a process of polarized division at specific sites determined by their cell types. In haploid organisms, such as *C. glabrata*, cell budding occurs in an axial manner, mother cells form new buds adjacent to previous bud site and daughter cells bud next to their birth site. However, as a cell ages, its budding pattern appears to be disrupted with a manifestation of budding randomly at a higher frequency (Wang et al., 2017). The deletion of *BUD27* in *S. cerevisiae* strains results in a 40% random budding pattern (Ni and Snyder, 2001). Furthermore, *ΔScbud27* mutants are slow growing and sensitive to translation inhibitors (Deplazes et al., 2009). *BUD27* was also described as part of a chaperone-network involved in cotranslational quality control (Deplazes et al., 2009). It is thought that this gene is involved in a feedback mechanism that actively prevents translation initiation under conditions when misfolded proteins accumulate (Deplazes et al., 2009). In a particular case, *CgBUD27* was found to be Pdr1-dependent in response to fluconazole, as in a mutant *PDR1* the expression of *CgBUD27* decreased considerably compared to the wild-type after exposure of the strain to fluconazole (Caudle, 2010). This curious observation allows us to question whether deletion in the gene will have any relation to fluconazole resistance. Since this gene is a translation regulator, which prevents translation initiation, it could be directly or indirectly related to the inhibition of the synthesis of some protein

involved in fluconazole resistance, perhaps involved in some mechanism of internalization of this drug. Simultaneously, it may also inhibit the synthesis of other proteins which impairs cell growth. It will be interesting to determine whether replacement of the mutant gene in the wild-type gene reverses the resistance phenotype. In fact, if we analyze the phenotypes of its orthologues in *S. cerevisiae*, their disruption results in an increased in fenpropimorph resistance (Kapitzky et al., 2010), a known antifungal drug and also an increase in bleomycin resistance (Kapitzky et al., 2010), an antimicrobial agent. However, its disruption also appears to confer resistance to a several chemical compounds, including some antifungal agents (table15). Since it has not yet been determined whether its disruption has influence on fluconazole resistance, this would be the next step in the study of the eventual role of this gene.

3.3.2.2 Candidate genes whose activity is likely to be fluconazole related

3.3.2.2.1 *CAGL0G00968g/VRP1*

The *CAGL0G00968g* gene has 15 orthologous genes in *Candida* species. Among them *C. albicans CaVRP1* and *S. cerevisiae ScVRP1* have been characterized (table 18). For the sake of simplicity, *CAGL0G00968g* will here be referred as *CgVRP1*.

Table 18: Description of Cg*VRP1* and orthologs in *S. cerevisiae* and *C. albicans*.

Strain	Designation	Description[a]
C. glabrata	*CAGL0G00968g*	Ortholog(s) have actin binding activity
S. cerevisiae	*VRP1*	Verprolin, proline-rich actin-associated protein; involved in cytoskeletal organization and cytokinesis; promotes actin nucleation and endocytosis; related to mammalian Wiskott-Aldrich syndrome protein (WASP)-interacting protein (WIP)
C. albicans	*VRP1*	Verprolin-related protein involved in actin cytoskeleton organization and polarized morphogenesis; interacts with Wal1 and Myo5; downregulated upon adherence to polystyrene

[a] Descriptions are based on those in the SGD (www.yeastgenome.org), and CGD (www.candidagenome.org).

CgVrp1 only has one identified motif (figure 23), WH2 (Wiskott-Aldrich syndrome homology region 2), which has been shown to be the region that interacts with actin (Machesky and Insall, 1998).It is responsible for controlling actin polymerization, and, as such, is important in cellular processes such as cell contractility, cell motility, cell trafficking and cell signaling (Veltman and Insall, 2010). The 2 insertions found on *CgVRP1* (figure 23) are not located in this identified motif (WH2).

Figure 23: Distribution of *CgVRP1* specific mutations found in the evolved resistant mutant *Δpdr1_2fr*. Mutations are indicated by red bars. WH2: WH2 (Wiskott-Aldrich syndrome homology region 2) motif. Domains are based on the data available in the CGD (www.candidagenome.org/).

When analyzing the effects of Arp2/3 complex deletion in *S. cerevisiae* severe defects on growth are observed, or more drastically lethality (Winter et al., 1999). The same was observed in *S. pombe* (Balasubramanian et al., 1996). *VRP1* is involved in the Arp2/3 complex activation. Curiously, it was verified that *Δpdr1_2fr* mutant shows growth defects (Marek and Korona, 2013; Yoshikawa et al., 2011). This phenotype may be, at least partially caused by the insertion of 2 amino acids on CgVrp1, assuming that this insertion affects negatively the activation of the Arp2/3 complex whose deletion results in severe defects on growth.

The question that now arises is what possible mechanisms the *VRP1* gene may be involved related to fluconazole resistance. Interestingly, *ScVRP1* was associated with resistance to antifugal drugs such as fluconazole (Kapitzky et al., 2010) sirolimus (Dudley et al., 2005) and flucytosine (Costa et al., 2015) and other variety of chemical compounds (table 19). Decreased resistance to fluconazole was observed in *S. cerevisiae* when *ScVRP1* was deleted (Kapitzky et al., 2010). On the contrary disruption of *VRP1* led to Caspofungin resistance in *S. cerevisiae* (Markovich et al., 2004). Given the observation that disruption of *VRP1* leads to a decrease in resistance to fluconazole in *S. cerevisiae*, it may be hypothesized that the predicted insertions in *CgVRP1* may lead to a gain in function in this gene. *VRP1* is involved in cell trafficking and cell signaling, therefore, it can be hypothesized to be involved in some export mechanism. Further studies will be needed to determine the relevance of this gene.

Table 19: Influence in chemical compounds resistance in *VRP1* deletion mutants of *S. cerevisiae*. Phenotypes based on those in the CGD (www.yeastgenome.org).

Class	Drug	Organism
		S. cerevisiae VRP1
Antifungal drug	Fluconazole	Decreased
	Sirolimus	Decreased
	Flucytosine	Decreased
	Caspofungin	Increased
Fungicide	Fenpropimorph	Decreased
	Cycloheximide	Decreased
	Sorbate	Decreased
Antifungal agent	Monensin A	Decreased
	Concanamycin A	Decreased
Antiseptic drug	Ethanol	Decreased
Anticancer agent	Cisplatin	Decreased
	Hydroxyurea	Decreased
	5-fluorouracil	Decreased
	Mycophenolic acid	Decreased
	Everolimus	Decreased
Antimicrobial agent	Bleomycin	Decreased
	Propolis extract	Decreased
	Valinomycin	Decreased
	Sulfanilamide	Decreased
	Hygromycin B	Decreased
Herbicide	Sulfometuron methyl	Decreased
Mutagen	Caffeine	Decreased
	Benzo[a]pyrene	Decreased
Antimalarial	Quinine	Decreased
	Mefloquine	Decreased
Toxin	Enniatin	Decreased

3.3.2.2.2 *CAGL0F04587g/PSY4*

PSY4 is the standard name of the *S. cerevisiae* ortholog gene of *CAGL0F04587g*. Among *Candida* species there are 11 more orthologs, including *C. albicans CaPSY4*. *ScPSY4* and *CaPSY4* are well-characterized and show similar functions (table 20). For the sake of simplicity, *CAGL0F04587g* will here be referred as *CgPSY4*.

Table 20: Description of *CgPSY4* and orthologs in *S. cerevisiae* and *C. albicans*.

Strain	Designation	Description[a]
C. glabrata	*CAGL0F04587g*	Ortholog(s) have protein phosphatase regulator activity, role in negative regulation of DNA damage checkpoint, protein dephosphorylation and cytoplasm, nucleus, protein phosphatase 4 complex localization
S. cerevisiae	*PSY4*	Regulatory subunit of protein phosphatase PP4; presence of Psy4 in the PP4 complex (along with catalytic subunit Pph3 and Psy2) is required for dephosphorylation of the histone variant H2AX, but not for dephosphorylation of Rad53, during recovery from the DNA damage checkpoint; localization is cell-cycle dependent and regulated by Cdc28 phosphorylation; required for cisplatin resistance; homolog of mammalian R2
C. albicans	*PSY4*	Regulatory subunit of protein phosphatase PP4; required for recovery from filamentation induced by DNA damage; mutants show increased virulence

[a] Descriptions are based on those in the SGD (www.yeastgenome.org), and CGD (www.proteingenome.org).

The mutation found in *CgPSY4* (figure 24) is located in an identified PPP4R2 (protein phosphatase 4 core regulatory subunit R2) domain. PPP4R2 is the regulatory subunit of the histone H2A phosphatase complex. Its function was first described in Hastie et al., 2006, where it has also been shown that this gene confers resistance to cisplatin (anticancer drug that binds to DNA) in *S. cerevisiae* (Hastie et al., 2006).

Figure 24: Distribution of *CgPSY4* specific mutations found in the evolved resistant mutant *Δpdr1_2fr*. Mutations are indicated by red bars. PPP4R2: protein phosphatase 4 core regulatory subunit R2. Domains are based on the data available in the CGD (www.candidagenome.org/).

When DNA damage occurs, the activation by phosphorylation of the checkpoint protein kinase Rad53 is fundamental for cells to enter checkpoint arrest, where remain until the defects are repaired. The deactivation by dephosphorylation of Rad53 is required for adaptation and recovery from DNA damage (Feng et al., 2017). PP4 is a protein phosphatase complex in eukaryotic cells that has been shown to play roles in the recovery and adaptation from DNA damage, that negatively regulates Rad53 (Cohen et al., 2005). In *S. cerevisiae*, the catalytic subunit, ScPph3 form a complex with the core regulatory subunit ScPsy4, and these complexes may further associate with a third variable regulatory subunit ScPsy2 (Cohen et al., 2005; Feng et al., 2017). The ScPph3-ScPsy2-ScPsy4 complex dephosphorylate histone γH2A resulting in the deactivation of Rad53 after checkpoint arrest (O'Neill et al., 2007). In *C. albicans* this mechanism is similar (Feng et al., 2017). However, deletion of *CaPSY4* causes *C. albicans* cells increased virulence and defect in DNA damage-induced filamentation (critical virulence factor in *C. albicans*) (Feng et al., 2017).

Curiously, *PSY4* seems to be related to resistance to diverse chemical compounds. In table 21 is summarized the effect on resistance to chemical compounds of *ScPSY4* and *CaPSY4* null mutants. Significantly, the *ScPSY4* mutant displays decreased fluconazole resistant (Kapitzky et al., 2010). Thus, *CgPSY4* may also play a role in resistance to this drug. Additionally, as shown in table 16, mutants of this gene also lead to a decrease in resistance to oxidative stress and zinc deficiency. This association with resistance to drugs and oxidative stress make *CgPSY4* a great candidate to be studied. Some reports have been published associating fluconazole with induction of ROS generation and oxidative DNA damage in *C. glabrata*. Mahl et al., 2015 demonstrated that subinhibitory concentrations of fluconazole can induce an increased generation of ROS and cause oxidative DNA damage (Mahl et al., 2015). Other case associating fluconazole with DNA damage in *C. tropicalis* was reported, although in combination with another drug (da Silva et al., 2013). Given the observation that fluconazole can induce DNA damage in *C. glabrata*, it can be hypothesized a possible connection between *CgPSY4* gene and a role in fluconazole resistance. *ScPSY4* and *CaPSY4* are involved in adaptation and recovery from DNA damage. Furthermore, these orthologs are associated to resistance to several chemical compounds, including fluconazole and other antifungal agents. The mutation here reported,

G313E, is located in the regulatory subunit of the complex responsible for the dephosphorylation of histone γH2A, and for adaptation and recovery from DNA damage, which gives more robustness to this hypothesis. For this reason, it appears reasonable to hypothesize that this mutation may lead to a higher *CgPSY4* activity, leading to azole resistance.

Table 21: Influence in chemical compounds resistance in *PSY4* null mutants of *S. cerevisiae* and *C. albicans*. Phenotypes based on those in the SGD (www.yeastgenome.org), and CGD (www.candidagenome.org).

Class	**Drug**	**Organism**	
		S. cerevisiae PSY4	***C. albicans PSY4***
Antifungal drug	Fluconazole	Decreased	-
	Sirolimus	Increased	-
Fungicide	Fenpropimorph	Decreased	-
	Cycloheximide	Increased	-
Antifungal agent	Phleomycin D1	Decreased	-
	Benzethonium chloride	Decreased	-
	Ascomycin	-	-
Anticancer agent	Cisplatin	Decreased	Decreased
	Hydroxyurea	-	Decreased
	Methyl methanesulfonate	-	Decreased
	Camptothecin	Decreased	-
	Epothilone	Decreased	-
Antimicrobial agent	Idarubicin	Decreased	-
	Bleomycin	Decreased	-
Pesticide	Glyoxal	Decreased	-

3.3.2.2.3 *CAGL0A01408g/PGD1*

PGD1 is the standard name of the *S. cerevisiae* ortholog gene of *CAGL0A01408g* (table 22). For the sake of simplicity *CAGL0A01408g* will here be referred as *CgPGD1*, despite not having an official standard name.

Table 22: Description of *CgPGD1*and the *S. cerevisiae* ortholog.

Strain	**Designation**	**Description[a]**
C. glabrata	*CAGL0A01408g*	Ortholog(s) have RNA polymerase II activating transcription factor binding, RNA polymerase II core promoter sequence-specific DNA binding and RNA polymerase II repressing transcription factor binding
S. cerevisiae	*PGD1*	Subunit of the RNA polymerase II mediator complex; associates with core polymerase subunits to form the RNA polymerase II holoenzyme; essential for basal and activated transcription; direct target of Cyc8-Tup1 transcriptional corepressor

[a] Descriptions are based on those in the SGD (www.yeastgenome.org), and CGD (www.candidagenome.org).

CgPGD1 encodes one of the subunits of the Mediator complex, Med3. The Mediator complex has an important role in transcription, interacting with the carboxy-terminal domain of the largest subunit of RNA polymerase II and acting as a bridge between upstream gene-specific regulatory proteins and core RNA polymerase II complex to activate target gene transcription (Casamassimi and Napoli, 2007). This complex is composed of three different modules, the head, middle, and tail. The largest of them, the tail, in *S. cerevisiae* is composed by the nonessential subunits Med2, Med3, Med14, Med15, and Med16 (Borah et al., 2014).

Interestingly, disruption of *CgMED2* and *CgPGD1* results in loss of viability during fluconazole stress in *C. glabrata* (Borah et al., 2011). Disruption of *CgPGD1* in a fluconazole resistant strain, harboring a *PDR1* GOF mutation, showed no effect in fluconazole resistance. However, in susceptible strains this gene was found to confer fluconazole resistance. Since our resistant mutant does not have the *PDR1* gene, the role of the *PGD1* gene may be very significant. Interestingly in Liu and Meyrs 2017, the deletion of the Med3 subunit in *C. albicans* was found to reduce the increased *MDR1* expression and fluconazole MIC conferred by *MRR1* GOF mutations (Liu and Myers, 2017). Since *PGD1* encodes the Med3 subunit, these findings suggest that this

gene could be involved in MFS transporters mechanisms of resistance and related to *MRR1*. It will be interesting to assess if the point mutation registered in *CgPGD1*, affecting the Med3 domain (Figure 25), leads to increased activity of the protein, thus conferring azole resistance. If this is the case, it will also be interesting to assess the impact of this form of CgMed3 in the transcriptome of *C. glabrata* cells, and maybe determine the expression of the *CgFLR1* gene, pointed out as the close homolog of *CaMDR1*.

Figure 25: Distribution of *CcPDG1* specific mutations found in the evolved resistant mutant *Δpdr1_2fr.* Mutations are indicated by red bars. Med3: Mediator complex subunit 3 fungal. Domains are based on the data available in the CGD (www.candidagenome.org/).

3.3.2.2.4 *CAGL0M14091g*

CAGL0M14091g encodes a putative quinone reductase/NADPH dehydrogenase. The mutation found (figure 26) is located in an identified flavodoxin-like fold domain. Flavodoxin-like fold family includes bacterial and eukaryotic quinone reductase/NADPH dehydrogenases. These enzymes use a flavin adenine dinucleotide (FAD) co-factor and catalyze the NAD(P)H-dependent two-electron reductions of quinones and protect cells against damage by free radicals and reactive oxygen species (Li et al., 1995). Putative quinone reductase/NADPH dehydrogenases were reported in bacteria as modulators of drug activity, protecting cells from stress mediated damage and conferring resistance to some drugs (Chatterjee and Sternberg, 1995; Hong et al., 2008).

Figure 26: Distribution of *CAGL0M14091g* mutations found in the involved resistant mutant *Δpdr1_2fr*. Mutations are indicated by red bars. FLF: Flavodoxin-like fold domain. Domains are based on the data available in the CGD (www.candidagenome.org/).

A hypothetical protein structure was obtained from the Top PDB Hit, a putative quinone reductase from *Klebsiella Pneumoniae* (PDB ID: 4GI5), 46% identical to Cagl0m14091g (figure 27). The 4GI5 aminoacids that bind to the cofactor FAD are known: HIS9, ARG63, ASP198, GLY155,

TYR160, TRP102, PHE103, LEU100, THR152. The mutation reported here, PRO158SER, that lies between GLY155 and TYR160, may be associated to the binding site of FAD co-factor, and likely to have an impact on its function, either limiting or inducing it.

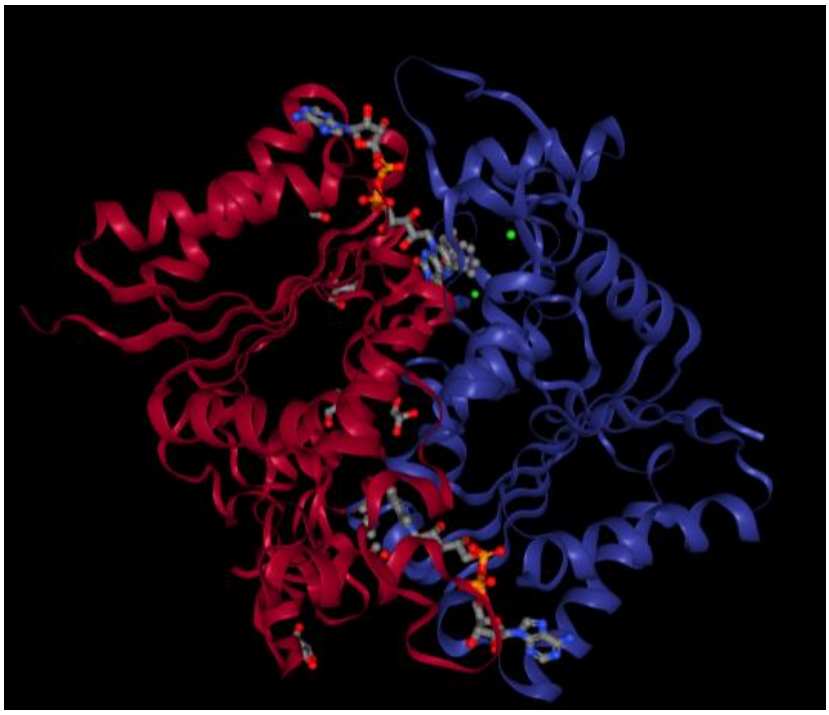


Figure 27: Crystal structure of a putative quinone reductase from *Klebsiella pneumoniae*, Top PDB Hit for Cagl0m14091g. PDB ID: 4GI5. Biological assembly assigned by authors and generated by PISA, image obtained through the Jmol software.

Interestingly, *CAGL0M14091g* may, in some way, be related to azoles resistance (Noble et al., 2013; Tsai et al., 2010; Vermitsky et al., 2006). Firstly, in Vermitsky et al., 2006, expression of *CAGL0M14091g* was found to be overexpressed in *C.glabrata* azole resistance clinical isolates (Vermitsky et al., 2006). Additionally, *CAGL0M14091g* was found to be up-regulated in a *C. glabrata PDR1* GOF resistant mutant relative to the parental 66032. Interestingly *CAGL0M14091g* was also upregulated in an *Candida glabrata* oropharyngeal azole resistant isolate, when compared to its parental azole susceptible isolate (Tsai et al., 2010). Data show that the *CAGL0M14091g* is regulated positively by CgPdr1 and negatively by CgStb5, a negative regulator of azole drug resistance genes. Therefore, it seems likely that *CAGL0M14091g* may play a role in fluconazole resistance, through a still unidentified mechanism. If this is the case and the registered point mutation leads to an increased activity of this protein, it might contribute to the fluconazole resistance phenotype observed in the *Δpdr1_2fr* strain. It will also be interesting to study whether this gene is responsible for fluconazole resistance.

3.3.2.3 Other potential candidate genes

3.3.2.3.1 *CAGL0M08492g/PIR3*

PIR3 has 14 orthologs in *Candida* species, including *C. albicans CaPIR1*, and one ortholog in *S. cerevisiae*, *ScPIR1*. These two orthologs show similar functions (table 23). The mutation A133T found in this gene (figure 28), cannot be attributed to a specific functional domain, as none has been described so far, except for a characteristic signal peptide.

Figure 28: Distribution of *CgPIR3* specific mutations found in the involved resistant mutant *Δpdr1_2fr*. Mutations are indicated by red bars. SP: signal peptide.

In *C. albicans* the Pir1 is responsible for cell wall maintenance, and has been demonstrated to be overexpressed in the cell wall upon long term fluconazole treatment (Nasrollahi et al., 2015). This protein seems to be attached to β-1,3-glucan covalently, acting as a glue to protect the yeast cell, being essential to maintain the integrity of *C. albicans* cell wall (Martinez et al., 2004; Nasrollahi et al., 2015). *PIR1* seems to have a critical role in cell wall formation, however, its mechanism of action is still unknown (Nasrollahi et al., 2015). Additionally, *S. cerevisiae* ScPir1 was also associated whit cell wall stability (Mrsa and Tanner, 1999). None of these proteins has been linked so far with fluconazole resistance. Nonetheless, it would be interesting to assess the eventual effect of the observed point mutation in *C. glabrata* resistance to fluconazole.

Table 23: Description of *CgPIR3* and orthologs in *S. cerevisiae* and *C. albicans*.

Strain	Designation	Description[a]
C. glabrata	*PIR3*	Pir protein family member, putative cell wall component
S. cerevisiae	*PIR1*	O-glycosylated protein required for cell wall stability; attached to the cell wall via beta-1,3-glucan; mediates mitochondrial translocation of Apn1; expression regulated by the cell integrity pathway and by Swi5 during the cell cycle
C. albicans	*PIR1*	1,3-beta-glucan-linked cell wall protein; N-mannosylated, O-glycosylated by Pmt1; cell wall defect in het mutant; Hog1/fluconazole/hypoxia induced; iron/Efg1/Plc1/temp regulated; flow model biofilm induced; hyphal, Spider biofilm repressed

[a] Descriptions are based on those in the SGD (www.yeastgenome.org), and CGD (www.candidagenome.org).

3.3.2.3.2 *CAGL0H09592g*

Although there is no clear ortholog of *CAGL0H09592g* in *S. cerevisiae*, the gene *ScTIR1* is pointed out as its close homolog. *CAGL0H09592g* encodes a 236 aminoacid putative GPI-linked cell wall protein, is predicted as a constituent of the cell wall involved in the stress response. The mutation identified in this study is located within a possible described domain involved in stress response (figure 29), belonging to the Srp1/Tip1 family. The proteins from this family are known to be induced by various stress conditions, glucose, cold-shock or temperature-shock.

Figure 29: Distribution of *CAGL0H09592g* specific mutations found in the evolved resistant mutant *Δpdr1_2fr*. Mutations are indicated by red bars. TIP1: Seripauperin and TIP1 family domain. PIR: Yeast PIR protein repeat. Domains are based on the data available in the CGD (www.candidagenome.org/).

It is known that *CAGL0H09592g* should be involved in the stress response, however, there are not many more studies on this protein. It may be interesting to study its possible role in fluconazole resistance given its role in stress resistance perhaps some function not yet described may be at the basis of the phenotype observable in the mutant.

3.3.2.3.3 *CAGL0M12617g/YRB2*

A premature stop codon was gained in gene *CAGL0M12617g* in the *Δpdr1_2fr* evolved mutant. Given the location of the premature stop within the functional domain of this protein (Seripauperin and TIP1 family domain) (figure 30), it is possible that it may lead to its inactivation. *CgYRB2* has 15 orthologs in *Candida* species, including *C. albicans*, and the well characterized *S. cerevisiae* orthologue *ScYRB2* (table 24).

Figure 30: Distribution of *CgYRB2* specific mutation found in the evolved resistant mutant *Δpdr1_2fr*. Mutations are indicated by red bars. RanBP1: Seripauperin and TIP1 family domain. Domains are based on the data available in the CGD (www.candidagenome.org/).

YRB1, *YRB2* and *NUP2* are the 3 genes of *S. cerevisiae* that encode proteins that contain the Ran-binding region (Taura et al., 1998). Ran hydrolyzes GTP which is required for nuclear transport, this enzyme defines the movement of macromolecules in both directions across the nuclear envelope (Melchior et al., 1993; Taura et al., 1998). To date, there is no reported relationship of this gene with fluconazole. However, given that this gene may have lost its function in our mutant, it might be interesting to determine whether re-insertion of the normal gene restored the susceptibility phenotype.

Table 24: Description of *CgYRB2* and orthologs in *S. cerevisiae* and *C. albicans*.

Strain	**Designation**	**Description[a]**
C. glabrata	*CAGL0M12617g*	Ortholog(s) have role in regulation of chromatin silencing at telomere, ribosomal small subunit export from nucleus and cytosol, nuclear pore, nucleus localization
S. cerevisiae	*YRB2*	Protein of unknown function; involved in nuclear processes of the Ran-GTPase cycle; involved in nuclear protein export; contains Ran Binding Domain and FxFG repeats; interacts with Srm1p, GTP-Gsp1, Rna1 and Crm1; relocalizes to the cytosol in response to hypoxia; not essential for viability
C. albicans	*C2_05530C_A*	Ortholog(s) have role in regulation of chromatin silencing at telomere, ribosomal small subunit export from nucleus and cytosol, nuclear pore, nucleus localization

[a] Descriptions are based on those in the SGD (www.yeastgenome.org), and CGD (www.candidagenome.org).

4. Conclusion

In this study, *C. glabrata* was shown to be able to acquire fluconazole resistance independently of the transcription factor Pdr1, a major regulator of drug resistance in this yeast.

In a small collection of clinical isolates, strains exhibiting fluconazole resistance were found to have, in 60% of the cases, GOF or GOF-like mutations in the *PDR1* sequence, which are likely to underlie the observed phenotype. However, in 40% of the cases, there were no GOF mutations in *PDR1*, suggesting that there are alternative mechanisms of azole resistance acquisition in the clinical setting. Looking for possible GOF mutations in two other transcription factors, Rpn4 and Mrr1, shown in our lab to confer azole resistance, it was possible to identify a mutation in the *RPN4* sequence, found to occur only in azole resistance strains, that we propose as a possible GOF mutation. It will be very interesting to analyze the impact of this specific mutation in Rpn4 activity and in fluconazole resistance. In vitro, we found that it is possible to evolve strains lacking important determinants of azole resistance towards a fluconazole resistance phenotype in just a few days, without acquiring GOF mutations in Pdr1 or displaying altered expression of the major fluconazole efflux pump, Cdr1. Particularly surprising was the finding that even a *Δpdr1* mutant strain can be forced to evolve towards fluconazole resistance. Given this, the genome of one of these evolved resistant mutants was sequenced in an attempt to reveal which mechanisms were behind this phenotype. The selected mutant showed growth difficulties comparing to the wild-type, so the changes responsible for acquisition of resistance may have led to a loss of fitness in the absence of the drug.

It has been found that even mutants without *PDR1* are able to acquire resistance to fluconazole. Genome sequencing enabled the identification of mutations in 36 genes, one or several of which must underlie the observed fluconazole resistance phenotype. Among the identified mutations, many were found to affect adhesin encoding genes. This type of proteins involved in cell adhesion may play an important role in preventing drug entry into cells, either by strengthening the cell envelop or by promoting cell-to-cell adhesion, decreasing the total area of cell surface exposed to the extracellular medium. One of these adhesins is encoded by *EPA3*, shown in our lab to confer azole drug resistance (Cavalheiro et al., unpublished results). Other genes in which mutations were found in the *Δpdr1_2fr* strain include *BUD27*, *PGD1*, *PSY4* and *VRP1*, whose orthologs in *S. cerevisiae* have been shown to confer multidrug resistance, the last two specifically against fluconazole. These mutations are considered the more likely to underlie the azole drug resistance phenotype observed in the *Δpdr1_2fr* strain, although the underlying mechanisms are yet to be understood. Several possible mechanisms have been proposed here, however, before proceeding to their confirmation, it will be interesting to test the biofilm formation capacity of the *Δpdr1_2fr,* measure its intercellular accumulation of fluconazole, and determine the expression of some transporters, namely the *CDR1* and the *FLR1* in order to reduce the number of

candidates on which further studies will focus. Additionally, it will be helpful to sequence the genome of the remaining *Δpdr1* strains evolved towards azole resistance. This will help us to narrow down the number of mutations that deserve further study, focusing on those that are common to all the evolved strains.

This study contributed to the discovery of new clues that will enable, in the near future, the elucidation of the mechanisms that allow *C. glabrata* to acquire fluconazole resistance independently of the transcription factor *PDR1* (Figure 31). This would be a great contribution for the understanding of azole drug resistance in this opportunistic pathogen, with expected impact in the design of new drugs and new therapeutic approaches to effectively treat candidiasis patients.

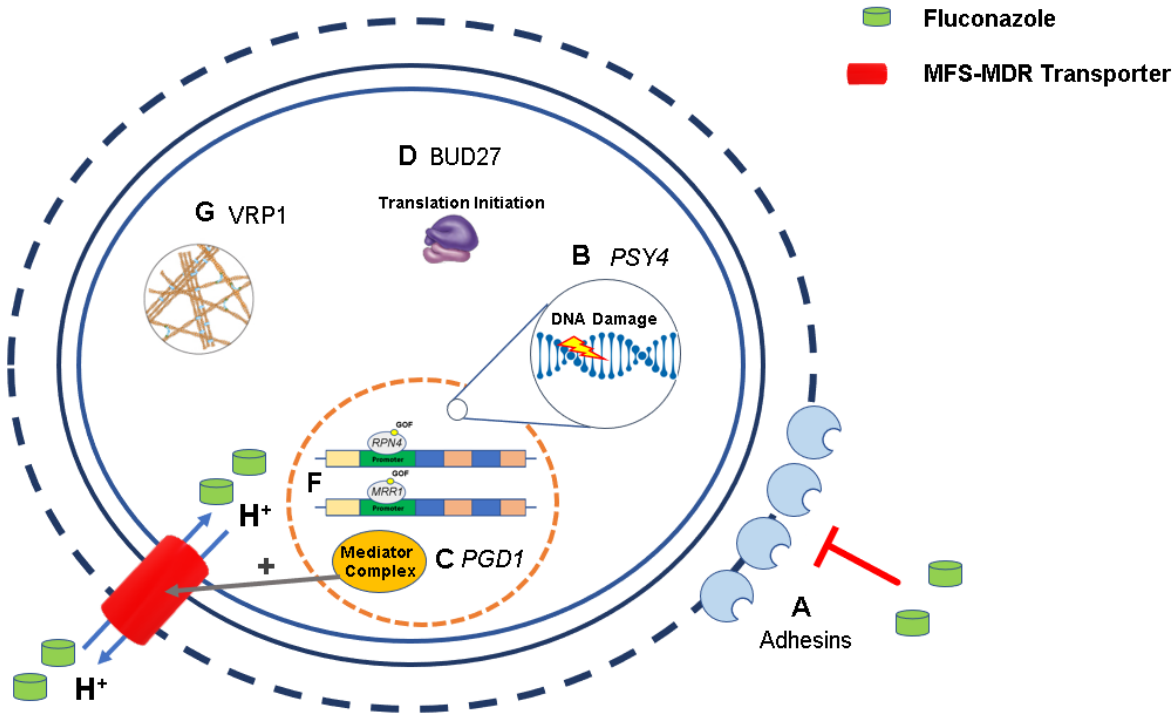


Figure 31: Model of the possible mechanisms of fluconazole resistance, beyond the Pdr1 network. A: Mutated adhesins, such as *EPA3*, may be a barrier to the entry of fluconazole into the cell. B: *PSY4* may be involved in the prevention of DNA damage caused by fluconazole. C: *PGD1* is a component of the mediator complex, likely to affect the expression of MFS-MDR transporters. D: Bud27 controls translation initiation, affecting drug resistance through unknown mechanisms. F: GOF mutations in the genes *RPN4* and *MRR1*. G: Vrp1 is involved with actin polymerization, drug resistance through unknown mechanisms.

5. References

Ahmad, K.M., Ishchuk, O.P., Hellborg, L., Jorgensen, G., Skvarc, M., Stenderup, J., Jorck-Ramberg, D., Polakova, S., and Piskur, J. (2013). Small chromosomes among Danish Candida glabrata isolates originated through different mechanisms (vol 104, pg 111, 2013). Anton Leeuw Int J G *104*, 281-282.

Alarco, A.M., and Raymond, M. (1999). The bZip transcription factor Cap1p is involved in multidrug resistance and oxidative stress response in Candida albicans. J Bacteriol *181*, 700-708.

Alcoba-Florez, J., Mendez-Alvarez, S., Cano, J., Guarro, J., Perez-Roth, E., and del Pilar Arevalo, M. (2005). Phenotypic and molecular characterization of Candida nivariensis sp. nov., a possible new opportunistic fungus. Journal of clinical microbiology *43*, 4107-4111.

Alexander, B.D., Johnson, M.D., Pfeiffer, C.D., Jimenez-Ortigosa, C., Catania, J., Booker, R., Castanheira, M., Messer, S.A., Perlin, D.S., and Pfaller, M.A. (2013). Increasing echinocandin resistance in Candida glabrata: clinical failure correlates with presence of FKS mutations and elevated minimum inhibitory concentrations. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America *56*, 1724-1732.

Allam, A., Kalnis, P., and Solovyev, V. (2015). Karect: accurate correction of substitution, insertion and deletion errors for next-generation sequencing data. Bioinformatics *31*, 3421-3428.

Ames, L., Duxbury, S., Pawlowska, B., Ho, H.L., Haynes, K., and Bates, S. (2017a). Galleria mellonella as a host model to study Candida glabrata virulence and antifungal efficacy. Virulence *8*, 1909-1917.

Ames, L., Duxbury, S., Pawlowska, B., Ho, H.L., Haynes, K., and Bates, S. (2017b). Galleria mellonella as a host model to study Candida glabrata virulence and antifungal efficacy. Virulence, 1-9.

Anderson, H.W. (1917). Yeast-like fungi of the human intestinal tract. J Infect Dis *21*, 341-U318.

Bader, O., Schwarz, A., Kraneveld, E.A., Tangwattanachuleeporn, M., Schmidt, P., Jacobsen, M.D., Gross, U., De Groot, P.W., and Weig, M. (2012). Gross karyotypic and phenotypic alterations among different progenies of the Candida glabrata CBS138/ATCC2001 reference strain. PloS one *7*, e52218.

Balasubramanian, M.K., Feoktistova, A., McCollum, D., and Gould, K.L. (1996). Fission yeast Sop2p: A novel and evolutionarily conserved protein that interacts with Arp3p and modulates profilin function. Embo J *15*, 6426-6437.

Barski, O.A., Tipparaju, S.M., and Bhatnagar, A. (2008). The aldo-keto reductase superfamily and its role in drug metabolism and detoxification. Drug metabolism reviews *40*, 553-624.

Ben-Ami, R., Zimmerman, O., Finn, T., Amit, S., Novikov, A., Wertheimer, N., Lurie-Weinberger, M., and Berman, J. (2016). Heteroresistance to Fluconazole Is a Continuously Distributed Phenotype among Candida glabrata Clinical Strains Associated with In Vivo Persistence. mBio *7*.

Benson, G. (1999). Tandem repeats finder: a program to analyze DNA sequences. Nucleic acids research *27*, 573-580.

Bergstrom, C.T., McElhany, P., and Real, L.A. (1999). Transmission bottlenecks as determinants of virulence in rapidly evolving pathogens. Proceedings of the National Academy of Sciences of the United States of America *96*, 5095-5100.

Bolger, A.M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics *30*, 2114-2120.

Bolotin-Fukuhara, M., and Fairhead, C. (2014). Candida glabrata: a deadly companion? Yeast *31*, 279-288.

Borah, S., Shivarathri, R., and Kaur, R. (2011). The Rho1 GTPase-activating protein CgBem2 is required for survival of azole stress in Candida glabrata. The Journal of biological chemistry *286*, 34311-34324.

Borah, S., Shivarathri, R., Srivastava, V.K., Ferrari, S., Sanglard, D., and Kaur, R. (2014). Pivotal role for a tail subunit of the RNA polymerase II mediator complex CgMed2 in azole tolerance and adherence in Candida glabrata. Antimicrobial agents and chemotherapy *58*, 5976-5986.

Bordallo-Cardona, M.A., Escribano, P., Marcos-Zambrano, L.J., Diaz-Garcia, J., de la Pedrosa, E.G., Canton, R., Bouza, E., and Guinea, J. (2017). Low and constant micafungin concentrations may be sufficient to lead to resistance mutations in FKS2 gene of Candida glabrata. Medical mycology.

Borst, A., Raimer, M.T., Warnock, D.W., Morrison, C.J., and Arthington-Skaggs, B.A. (2005). Rapid acquisition of stable azole resistance by Candida glabrata isolates obtained before the clinical introduction of fluconazole. Antimicrobial agents and chemotherapy *49*, 783-787.

Bouchara, J.P., Zouhair, R., Le Boudouil, S., Renier, G., Filmon, R., Chabasse, D., Hallet, J.N., and Defontaine, A. (2000). In-vivo selection of an azole-resistant petite mutant of Candida glabrata. J Med Microbiol *49*, 977-984.

Bouma, J.E., and Lenski, R.E. (1988). Evolution of a bacteria/plasmid association. Nature *335*, 351-352.

Brun, S., Berges, T., Poupard, P., Vauzelle-Moreau, C., Renier, G., Chabasse, D., and Bouchara, J.P. (2004). Mechanisms of azole resistance in petite mutants of Candida glabrata. Antimicrobial agents and chemotherapy *48*, 1788-1796.

Brunke, S., Seider, K., Fischer, D., Jacobsen, I.D., Kasper, L., Jablonowski, N., Wartenberg, A., Bader, O., Enache-Angoulvant, A., Schaller, M.*, et al.* (2014). One small step for a yeast--microevolution within macrophages renders Candida glabrata hypervirulent due to a single point mutation. PLoS pathogens *10*, e1004478.

Casamassimi, A., and Napoli, C. (2007). Mediator complexes and eukaryotic transcription regulation: an overview. Biochimie *89*, 1439-1446.

Caudle, K.E., Barker, K.S., Wiederhold, N.P., Xu, L., Homayouni, R., and Rogers, P.D. (2011). Genomewide expression profile analysis of the Candida glabrata Pdr1 regulon. Eukaryotic cell *10*, 373-383.

Chatterjee, P.K., and Sternberg, N.L. (1995). A general genetic approach in Escherichia coli for determining the mechanism(s) of action of tumoricidal agents: application to DMP 840, a tumoricidal agent. Proceedings of the National Academy of Sciences of the United States of America *92*, 8950-8954.

Cho, E.J., Shin, J.H., Kim, S.H., Kim, H.K., Park, J.S., Sung, H., Kim, M.N., and Im, H.J. (2015). Emergence of multiple resistance profiles involving azoles, echinocandins and amphotericin B in Candida glabrata isolates from a neutropenia patient with prolonged fungaemia. The Journal of antimicrobial chemotherapy *70*, 1268-1270.

Cingolani, P., Platts, A., Wang le, L., Coon, M., Nguyen, T., Wang, L., Land, S.J., Lu, X., and Ruden, D.M. (2012). A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. Fly *6*, 80-92.

CLSI (2012). Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts. National Committee for Clinical Laboratory Standards, Wayne, Pa.

Cohen, P.T., Philp, A., and Vazquez-Martin, C. (2005). Protein phosphatase 4--from obscurity to vital functions. FEBS letters *579*, 3278-3286.

Cormack, B.P., Ghori, N., and Falkow, S. (1999). An adhesin of the yeast pathogen Candida glabrata mediating adherence to human epithelial cells. Science *285*, 578-582.

Correia, A., Sampaio, P., James, S., and Pais, C. (2006). Candida bracarensis sp. nov., a novel anamorphic yeast species phenotypically similar to Candida glabrata. International journal of systematic and evolutionary microbiology *56*, 313-317.

Costa, C., Nunes, J., Henriques, A., Mira, N.P., Nakayama, H., Chibana, H., and Teixeira, M.C. (2014). Candida glabrata drug:H+ antiporter CgTpo3 (ORF CAGL0I10384g): role in azole drug resistance and polyamine homeostasis. The Journal of antimicrobial chemotherapy *69*, 1767-1776.

Costa, C., Ponte, A., Pais, P., Santos, R., Cavalheiro, M., Yaguchi, T., Chibana, H., and Teixeira, M.C. (2015). New Mechanisms of Flucytosine Resistance in C. glabrata Unveiled by a Chemogenomics Analysis in S. cerevisiae. PloS one *10*, e0135110.

Costa, C., Ribeiro, J., Miranda, I.M., Silva-Dias, A., Cavalheiro, M., Costa-de-Oliveira, S., Rodrigues, A.G., and Teixeira, M.C. (2016). Clotrimazole Drug Resistance in Candida glabrata Clinical Isolates Correlates with Increased Expression of the Drug:H(+) Antiporters CgAqr1, CgTpo1_1, CgTpo3, and CgQdr2. Front Microbiol *7*, 526.

Costerton, J.W., Stewart, P.S., and Greenberg, E.P. (1999). Bacterial biofilms: a common cause of persistent infections. Science *284*, 1318-1322.

Cowen, L.E. (2001). Predicting the emergence of resistance to antifungal drugs. FEMS microbiology letters *204*, 1-7.

Cowen, L.E., Anderson, J.B., and Kohn, L.M. (2002). Evolution of drug resistance in Candida albicans. Annual review of microbiology *56*, 139-165.

Cowen, L.E., Kohn, L.M., and Anderson, J.B. (2001). Divergence in fitness and evolution of drug resistance in experimental populations of Candida albicans. J Bacteriol *183*, 2971-2978.

Cowen, L.E., Sanglard, D., Calabrese, D., Sirjusingh, C., Anderson, J.B., and Kohn, L.M. (2000). Evolution of drug resistance in experimental populations of Candida albicans. J Bacteriol *182*, 1515-1522.

Cowen, L.E., and Steinbach, W.J. (2008). Stress, drugs, and evolution: the role of cellular signaling in fungal drug resistance. Eukaryotic cell *7*, 747-764.

da Silva, C.R., de Andrade Neto, J.B., Sidrim, J.J., Angelo, M.R., Magalhaes, H.I., Cavalcanti, B.C., Brilhante, R.S., Macedo, D.S., de Moraes, M.O., Lobo, M.D*., et al.* (2013). Synergistic effects of amiodarone and fluconazole on Candida tropicalis resistant to fluconazole. Antimicrobial agents and chemotherapy *57*, 1691-1700.

de Groot, P.W., Kraneveld, E.A., Yin, Q.Y., Dekker, H.L., Gross, U., Crielaard, W., de Koster, C.G., Bader, O., Klis, F.M., and Weig, M. (2008). The cell wall of the human pathogen Candida glabrata: differential incorporation of novel adhesin-like wall proteins. Eukaryotic cell *7*, 1951-1964.

Deplazes, A., Mockli, N., Luke, B., Auerbach, D., and Peter, M. (2009). Yeast Uri1p promotes translation initiation and may provide a link to cotranslational quality control. Embo J *28*, 1429-1441.

Desalermos, A., Fuchs, B.B., and Mylonakis, E. (2012). Selecting an invertebrate model host for the study of fungal pathogenesis. PLoS pathogens *8*, e1002451.

Diaz-Guerra, T.M., Mellado, E., Cuenca-Estrella, M., and Rodriguez-Tudela, J.L. (2003). A point mutation in the 14alpha-sterol demethylase gene cyp51A contributes to itraconazole resistance in Aspergillus fumigatus. Antimicrobial agents and chemotherapy *47*, 1120-1124.

Douglas, L.J. (2003). Candida biofilms and their role in infection. Trends in microbiology *11*, 30-36.

Dudley, A.M., Janse, D.M., Tanay, A., Shamir, R., and Church, G.M. (2005). A global view of pleiotropy and phenotypically derived gene function in yeast. Molecular systems biology *1*, 2005 0001.

Dujon, B., Sherman, D., Fischer, G., Durrens, P., Casaregola, S., Lafontaine, I., De Montigny, J., Marck, C., Neuveglise, C., Talla, E.*, et al.* (2004). Genome evolution in yeasts. Nature *430*, 35-44.

Dunkel, N., Liu, T.T., Barker, K.S., Homayouni, R., Morschhauser, J., and Rogers, P.D. (2008). A gain-of-function mutation in the transcription factor Upc2p causes upregulation of ergosterol biosynthesis genes and increased fluconazole resistance in a clinical Candida albicans isolate. Eukaryotic cell *7*, 1180-1190.

El-Halfawy, O.M., and Valvano, M.A. (2013). Chemical communication of antibiotic resistance by a highly resistant subpopulation of bacterial cells. PloS one *8*, e68874.

El-Halfawy, O.M., and Valvano, M.A. (2015). Antimicrobial heteroresistance: an emerging field in need of clarity. Clinical microbiology reviews *28*, 191-207.

Fan-Havard, P., Capano, D., Smith, S.M., Mangia, A., and Eng, R.H. (1991). Development of resistance in candida isolates from patients receiving prolonged antifungal therapy. Antimicrobial agents and chemotherapy *35*, 2302-2305.

Farahyar, S., Zaini, F., Kordbacheh, P., Rezaie, S., Safara, M., Raoofian, R., and Heidari, M. (2013). Overexpression of aldo-keto-reductase in azole-resistant clinical isolates of Candida glabrata determined by cDNA-AFLP. Daru : journal of Faculty of Pharmacy, Tehran University of Medical Sciences *21*, 1.

Farmakiotis, D., Tarrand, J.J., and Kontoyiannis, D.P. (2014). Drug-resistant Candida glabrata infection in cancer patients. Emerging infectious diseases *20*, 1833-1840.

Feng, J., Duan, Y., Qin, Y., Sun, W., Zhuang, Z., Zhu, D., and Jiang, L. (2017). The N-terminal pY33XL motif of CaPsy2 is critical for the function of protein phosphatase 4 in CaRad53 deactivation, DNA damage-induced filamentation and virulence in Candida albicans. International journal of medical microbiology : IJMM *307*, 471-480.

Ferrari, S., Ischer, F., Calabrese, D., Posteraro, B., Sanguinetti, M., Fadda, G., Rohde, B., Bauser, C., Bader, O., and Sanglard, D. (2009). Gain of function mutations in CgPDR1 of Candida glabrata not only mediate antifungal resistance but also enhance virulence. PLoS pathogens *5*, e1000268.

Ferrari, S., Sanguinetti, M., De Bernardis, F., Torelli, R., Posteraro, B., Vandeputte, P., and Sanglard, D. (2011a). Loss of mitochondrial functions associated with azole resistance in Candida glabrata results in enhanced virulence in mice. Antimicrobial agents and chemotherapy *55*, 1852-1860.

Ferrari, S., Sanguinetti, M., Torelli, R., Posteraro, B., and Sanglard, D. (2011b). Contribution of CgPDR1-regulated genes in enhanced virulence of azole-resistant Candida glabrata. PloS one *6*, e17589.

Fidel, P.L., Jr., Vazquez, J.A., and Sobel, J.D. (1999). Candida glabrata: review of epidemiology, pathogenesis, and clinical disease with comparison to C. albicans. Clinical microbiology reviews *12*, 80-96.

Fitzpatrick, D.A. (2012). Horizontal gene transfer in fungi. FEMS microbiology letters *329*, 1-8.

Gabaldon, T., and Carrete, L. (2016). The birth of a deadly yeast: tracing the evolutionary emergence of virulence traits in Candida glabrata. Fems Yeast Res *16*, fov110.

Garland, T., and Rose, M.R. (2009). Experimental evolution : concepts, methods, and applications of selection experiments (Berkeley: University of California Press).

Goffeau, A., Barrell, B.G., Bussey, H., Davis, R.W., Dujon, B., Feldmann, H., Galibert, F., Hoheisel, J.D., Jacq, C., Johnston, M.*, et al.* (1996). Life with 6000 genes. Science *274*, 546, 563-547.

Gohar, A.A., Badali, H., Shokohi, T., Nabili, M., Amirrajab, N., and Moazeni, M. (2017). Expression Patterns of ABC Transporter Genes in Fluconazole-Resistant Candida glabrata. Mycopathologia *182*, 273-284.

Harrison, B.D., Hashemi, J., Bibi, M., Pulver, R., Bavli, D., Nahmias, Y., Wellington, M., Sapiro, G., and Berman, J. (2014). A tetraploid intermediate precedes aneuploid formation in yeasts exposed to fluconazole. PLoS biology *12*, e1001815.

Hastie, C.J., Vazquez-Martin, C., Philp, A., Stark, M.J., and Cohen, P.T. (2006). The Saccharomyces cerevisiae orthologue of the human protein phosphatase 4 core regulatory subunit R2 confers resistance to the anticancer drug cisplatin. The FEBS journal *273*, 3322-3334.

Healey, K.R., Jimenez Ortigosa, C., Shor, E., and Perlin, D.S. (2016a). Genetic Drivers of Multidrug Resistance in Candida glabrata. Front Microbiol *7*, 1995.

Healey, K.R., Zhao, Y., Perez, W.B., Lockhart, S.R., Sobel, J.D., Farmakiotis, D., Kontoyiannis, D.P., Sanglard, D., Taj-Aldeen, S.J., Alexander, B.D.*, et al.* (2016b). Prevalent mutator genotype identified in fungal pathogen Candida glabrata promotes multi-drug resistance. Nature communications *7*, 11128.

Hitchcock, C.A. (1991). Cytochrome P-450-dependent 14 alpha-sterol demethylase of Candida albicans and its interaction with azole antifungals. Biochemical Society transactions *19*, 782-787.

Hong, Y., Wang, G., and Maier, R.J. (2008). The NADPH quinone reductase MdaB confers oxidative stress resistance to Helicobacter hepaticus. Microbial pathogenesis *44*, 169-174.

Hoot, S.J., Smith, A.R., Brown, R.P., and White, T.C. (2011). An A643V amino acid substitution in Upc2p contributes to azole resistance in well-characterized clinical isolates of Candida albicans. Antimicrobial agents and chemotherapy *55*, 940-942.

Hosogaya, N., Miyazaki, T., Nagi, M., Tanabe, K., Minematsu, A., Nagayoshi, Y., Yamauchi, S., Nakamura, S., Imamura, Y., Izumikawa, K.*, et al.* (2013). The heme-binding protein Dap1 links iron homeostasis to azole resistance via the P450 protein Erg11 in Candida glabrata. Fems Yeast Res *13*, 411-421.

Hou, X., Xiao, M., Wang, H., Yu, S.Y., Zhang, G., Zhao, Y., and Xu, Y.C. (2018). Profiling of PDR1 and MSH2 in Candida glabrata Bloodstream Isolates from a Multicenter Study in China. Antimicrobial agents and chemotherapy *62*.

Hwang, S., Kim, E., Lee, I., and Marcotte, E.M. (2015). Systematic comparison of variant calling pipelines using gold standard personal exome variants. Sci Rep-Uk *5*.

Ji, H., Zhang, W., Zhou, Y., Zhang, M., Zhu, J., Song, Y., and Lu, J. (2000). A three-dimensional model of lanosterol 14alpha-demethylase of Candida albicans and its interaction with azole antifungals. Journal of medicinal chemistry *43*, 2493-2505.

Johnson, E.M., Warnock, D.W., Luker, J., Porter, S.R., and Scully, C. (1995). Emergence of azole drug resistance in Candida species from HIV-infected patients receiving prolonged fluconazole therapy for oral candidosis. The Journal of antimicrobial chemotherapy *35*, 103-114.

Jones, T., Federspiel, N.A., Chibana, H., Dungan, J., Kalman, S., Magee, B.B., Newport, G., Thorstenson, Y.R., Agabian, N., Magee, P.T.*, et al.* (2004). The diploid genome sequence of Candida albicans. Proceedings of the National Academy of Sciences of the United States of America *101*, 7329-7334.

Ju, D., Wang, X., Ha, S.W., Fu, J., and Xie, Y. (2010). Inhibition of proteasomal degradation of rpn4 impairs nonhomologous end-joining repair of DNA double-strand breaks. PloS one *5*, e9877.

Kanafani, Z.A., and Perfect, J.R. (2008). Resistance to antifungal agents: Mechanisms and clinical impact. Clinical Infectious Diseases *46*, 120-128.

Kapitzky, L., Beltrao, P., Berens, T.J., Gassner, N., Zhou, C., Wuster, A., Wu, J., Babu, M.M., Elledge, S.J., Toczyski, D.*, et al.* (2010). Cross-species chemogenomic profiling reveals evolutionarily conserved drug mode of action. Molecular systems biology *6*, 451.

Karpov, D.S., Grineva, E.N., Leinsoo, A.T., Nadolinskaia, N.I., Danilenko, N.K., Tutyaeva, V.V., Spasskaya, D.S., Preobrazhenskaya, O.V., Lysov, Y.P., and Karpov, V.L. (2017). Functional analysis of Debaryomyces hansenii Rpn4 on a genetic background of Saccharomyces cerevisiae. Fems Yeast Res *17*.

Katiyar, S., Shiffrin, E., Shelton, C., Healey, K., Vermitsky, J.P., and Edlind, T. (2016). Evaluation of Polymorphic Locus Sequence Typing for Candida glabrata Epidemiology. Journal of clinical microbiology *54*, 1042-1050.

Kelly, S.L., Lamb, D.C., Kelly, D.E., Manning, N.J., Loeffler, J., Hebart, H., Schumacher, U., and Einsele, H. (1997). Resistance to fluconazole and cross-resistance to amphotericin B in Candida albicans from AIDS patients caused by defective sterol delta5,6-desaturation. FEBS letters *400*, 80-82.

Klimova, N., Yeung, R., Kachurina, N., and Turcotte, B. (2014). Phenotypic analysis of a family of transcriptional regulators, the zinc cluster proteins, in the human fungal pathogen Candida glabrata. G3 *4*, 931-940.

Kuhn, D.M., and Vyas, V.K. (2012). The Candida glabrata adhesin Epa1p causes adhesion, phagocytosis, and cytokine secretion by innate immune cells. Fems Yeast Res *12*, 398-414.

Kurtzman, C.P. (2003). Phylogenetic circumscription of Saccharomyces, Kluyveromyces and other members of the Saccharomycetaceae, and the proposal of the new genera Lachancea, Nakaseomyces, Naumovia, Vanderwaltozyma and Zygotorulaspora. Fems Yeast Res *4*, 233-245.

Lenski, R.E., and Travisano, M. (1994). Dynamics of adaptation and diversification: a 10,000-generation experiment with bacterial populations. Proceedings of the National Academy of Sciences of the United States of America *91*, 6808-6814.

Levin, B.R., Perrot, V., and Walker, N. (2000). Compensatory mutations, antibiotic resistance and the population genetics of adaptive evolution in bacteria. Genetics *154*, 985-997.

Levy, S.B. (1994). Balancing the drug-resistance equation. Trends in microbiology *2*, 341-342.

Li, H., and Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics *25*, 1754-1760.

Li, R., Bianchet, M.A., Talalay, P., and Amzel, L.M. (1995). The three-dimensional structure of NAD(P)H:quinone reductase, a flavoprotein involved in cancer chemoprotection and chemotherapy: mechanism of the two-electron reduction. Proceedings of the National Academy of Sciences of the United States of America *92*, 8846-8850.

Li, Z.H., Lee, I., Moradi, E., Hung, N.J., Johnson, A.W., and Marcotte, E.M. (2009). Rational Extension of the Ribosome Biogenesis Pathway Using Network-Guided Genetics. PLoS biology *7*.

Liu, Z., and Myers, L.C. (2017). Candida albicans Swi/Snf and Mediator Complexes Differentially Regulate Mrr1-Induced MDR1 Expression and Fluconazole Resistance. Antimicrobial agents and chemotherapy *61*.

Lupetti, A., Danesi, R., Campa, M., Del Tacca, M., and Kelly, S. (2002). Molecular basis of resistance to azole antifungals. Trends in molecular medicine *8*, 76-81.

Ma, B., Pan, S.J., Zupancic, M.L., and Cormack, B.P. (2007). Assimilation of NAD(+) precursors in Candida glabrata. Molecular microbiology *66*, 14-25.

Ma, M., and Liu, Z.L. (2010). Comparative transcriptome profiling analyses during the lag phase uncover YAP1, PDR1, PDR3, RPN4, and HSF1 as key regulatory genes in genomic adaptation to the lignocellulose derived inhibitor HMF for Saccharomyces cerevisiae. BMC genomics *11*, 660.

Machesky, L.M., and Insall, R.H. (1998). Scar1 and the related Wiskott-Aldrich syndrome protein, WASP, regulate the actin cytoskeleton through the Arp2/3 complex. Current biology : CB *8*, 1347-1356.

Mah, T.F., and O'Toole, G.A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. Trends in microbiology *9*, 34-39.

Mahl, C.D., Behling, C.S., Hackenhaar, F.S., de Carvalho e Silva, M.N., Putti, J., Salomon, T.B., Alves, S.H., Fuentefria, A., and Benfato, M.S. (2015). Induction of ROS generation by fluconazole in Candida glabrata: activation of antioxidant enzymes and oxidative DNA damage. Diagnostic microbiology and infectious disease *82*, 203-208.

Mann, P.A., McNicholas, P.M., Chau, A.S., Patel, R., Mendrick, C., Ullmann, A.J., Cornely, O.A., Patino, H., and Black, T.A. (2009). Impact of antifungal prophylaxis on colonization and azole susceptibility of Candida species. Antimicrobial agents and chemotherapy *53*, 5026-5034.

Mann, P.A., Parmegiani, R.M., Wei, S.Q., Mendrick, C.A., Li, X., Loebenberg, D., DiDomenico, B., Hare, R.S., Walker, S.S., and McNicholas, P.M. (2003). Mutations in Aspergillus fumigatus resulting in reduced susceptibility to posaconazole appear to be restricted to a single amino acid in the cytochrome P450 14alpha-demethylase. Antimicrobial agents and chemotherapy *47*, 577-581.

Mansfield, B.E., Oltean, H.N., Oliver, B.G., Hoot, S.J., Leyde, S.E., Hedstrom, L., and White, T.C. (2010). Azole drugs are imported by facilitated diffusion in Candida albicans and other pathogenic fungi. PLoS pathogens *6*, e1001126.

Marcais, G., and Kingsford, C. (2011). A fast, lock-free approach for efficient parallel counting of occurrences of k-mers. Bioinformatics *27*, 764-770.

Marek, A., and Korona, R. (2013). Restricted pleiotropy facilitates mutational erosion of major life-history traits. Evolution; international journal of organic evolution *67*, 3077-3086.

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

Martel, C.M., Parker, J.E., Bader, O., Weig, M., Gross, U., Warrilow, A.G., Rolley, N., Kelly, D.E., and Kelly, S.L. (2010). Identification and characterization of four azole-resistant erg3 mutants of Candida albicans. Antimicrobial agents and chemotherapy *54*, 4527-4533.

Martinez, A.I., Castillo, L., Garcera, A., Elorza, M.V., Valentin, E., and Sentandreu, R. (2004). Role of Pir1 in the construction of the Candida albicans cell wall. Microbiology *150*, 3151-3161.

Maurelli, A.T. (2007). Black holes, antivirulence genes, and gene inactivation in the evolution of bacterial pathogens. FEMS microbiology letters *267*, 1-8.

McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., Garimella, K., Altshuler, D., Gabriel, S., Daly, M.*, et al.* (2010). The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome research *20*, 1297-1303.

Melchior, F., Paschal, B., Evans, J., and Gerace, L. (1993). Inhibition of nuclear protein import by nonhydrolyzable analogues of GTP and identification of the small GTPase Ran/TC4 as an essential transport factor. The Journal of cell biology *123*, 1649-1659.

Mindnich, R.D., and Penning, T.M. (2009). Aldo-keto reductase (AKR) superfamily: genomics and annotation. Human genomics *3*, 362-370.

Morio, F., Pagniez, F., Besse, M., Gay-andrieu, F., Miegeville, M., and Le Pape, P. (2013). Deciphering azole resistance mechanisms with a focus on transcription factor-encoding genes TAC1, MRR1 and UPC2 in a set of fluconazole-resistant clinical isolates of Candida albicans. International journal of antimicrobial agents *42*, 410-415.

Morschhauser, J., Barker, K.S., Liu, T.T., Bla, B.W.J., Homayouni, R., and Rogers, P.D. (2007). The transcription factor Mrr1p controls expression of the MDR1 efflux pump and mediates multidrug resistance in Candida albicans. PLoS pathogens *3*, e164.

Morschhauser, J., Kohler, G., Ziebuhr, W., Blum-Oehler, G., Dobrindt, U., and Hacker, J. (2000). Evolution of microbial pathogens. Philos T Roy Soc B *355*, 695-704.

Mrsa, V., and Tanner, W. (1999). Role of NaOH-extractable cell wall proteins Ccw5p, Ccw6p, Ccw7p and Ccw8p (members of the Pir protein family) in stability of the Saccharomyces cerevisiae cell wall. Yeast *15*, 813-820.

Musser, J.M. (1995). Antimicrobial agent resistance in mycobacteria: molecular genetic insights. Clinical microbiology reviews *8*, 496-514.

Narasimhan, V., Danecek, P., Scally, A., Xue, Y., Tyler-Smith, C., and Durbin, R. (2016). BCFtools/RoH: a hidden Markov model approach for detecting autozygosity from next-generation sequencing data. Bioinformatics *32*, 1749-1751.

Nasrollahi, Z., Yadegari, M.H., Roudbar Mohammadi, S., Roudbary, M., Hosseini Poor, M., Nikoomanesh, F., and Rajabi Bazl, M. (2015). Fluconazole Resistance Candida albicans in Females With Recurrent Vaginitis and Pir1 Overexpression. Jundishapur journal of microbiology *8*, e21468.

Ni, L., and Snyder, M. (2001). A genomic study of the bipolar bud site selection pattern in Saccharomyces cerevisiae. Molecular biology of the cell *12*, 2147-2170.

Ni, Q., Wang, C., Tian, Y., Dong, D., Jiang, C., Mao, E., and Peng, Y. (2018). CgPDR1 gain-of-function mutations lead to azole-resistance and increased adhesion in clinical Candida glabrata strains. Mycoses *61*, 430-440.

Nicholson, R.L., Butler, L.G., and Asquith, T.N. (1986). Glycoproteins from Colletotrichum-Graminicola That Bind Phenols - Implications for Survival and Virulence of Phytopathogenic Fungi. Phytopathology *76*, 1315-1318.

Nijhuis, M., Schuurman, R., de Jong, D., Erickson, J., Gustchina, E., Albert, J., Schipper, P., Gulnik, S., and Boucher, C.A. (1999). Increased fitness of drug resistant HIV-1 protease as a result of acquisition of compensatory mutations during suboptimal therapy. Aids *13*, 2349-2359.

Noble, J.A., Tsai, H.F., Suffis, S.D., Su, Q., Myers, T.G., and Bennett, J.E. (2013). STB5 is a negative regulator of azole resistance in Candida glabrata. Antimicrobial agents and chemotherapy *57*, 959-967.

O'Neill, B.M., Szyjka, S.J., Lis, E.T., Bailey, A.O., Yates, J.R., 3rd, Aparicio, O.M., and Romesberg, F.E. (2007). Pph3-Psy2 is a phosphatase complex required for Rad53 dephosphorylation and replication fork restart during recovery from DNA damage. Proceedings of the National Academy of Sciences of the United States of America *104*, 9290-9295.

Orozco, A.S., Higginbotham, L.M., Hitchcock, C.A., Parkinson, T., Falconer, D., Ibrahim, A.S., Ghannoum, M.A., and Filler, S.G. (1998). Mechanism of fluconazole resistance in Candida krusei. Antimicrobial agents and chemotherapy *42*, 2645-2649.

Pappas, P.G., Rex, J.H., Lee, J., Hamill, R.J., Larsen, R.A., Powderly, W., Kauffman, C.A., Hyslop, N., Mangino, J.E., Chapman, S.*, et al.* (2003). A prospective observational study of candidemia: epidemiology, therapy, and influences on mortality in hospitalized adult and pediatric patients. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America *37*, 634-643.

Perfect, S.E., O'Connell, R.J., Green, E.F., Doering-Saad, C., and Green, J.R. (1998). Expression cloning of a fungal proline-rich glycoprotein specific to the biotrophic interface formed in the Colletotrichum-bean interaction. The Plant journal : for cell and molecular biology *15*, 273-279.

Perlroth, J., Choi, B., and Spellberg, B. (2007). Nosocomial fungal infections: epidemiology, diagnosis, and treatment. Medical mycology *45*, 321-346.

Pfaller, M.A., Castanheira, M., Lockhart, S.R., Ahlquist, A.M., Messer, S.A., and Jones, R.N. (2012). Frequency of Decreased Susceptibility and Resistance to Echinocandins among Fluconazole-Resistant Bloodstream Isolates of Candida glabrata. Journal of clinical microbiology *50*, 1199-1203.

Pfaller, M.A., and Diekema, D.J. (2007). Epidemiology of invasive candidiasis: a persistent public health problem. Clinical microbiology reviews *20*, 133-163.

Pfaller, M.A., Messer, S.A., Hollis, R.J., Jones, R.N., and Diekema, D.J. (2002). In vitro activities of ravuconazole and voriconazole compared with those of four approved systemic antifungal agents against 6,970 clinical isolates of Candida spp. Antimicrobial agents and chemotherapy *46*, 1723-1727.

Polakova, S., Blume, C., Zarate, J.A., Mentel, M., Jorck-Ramberg, D., Stenderup, J., and Piskur, J. (2009). Formation of new chromosomes as a virulence mechanism in yeast Candida glabrata. Proceedings of the National Academy of Sciences of the United States of America *106*, 2688-2693.

Prasad, T., Chandra, A., Mukhopadhyay, C.K., and Prasad, R. (2006). Unexpected link between iron and drug resistance of Candida spp.: Iron depletion enhances membrane fluidity and drug diffusion, leading to drug-susceptible cells. Antimicrobial agents and chemotherapy *50*, 3597-3606.

Rainey, P.B., and Travisano, M. (1998). Adaptive radiation in a heterogeneous environment. Nature *394*, 69-72.

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

Redding, S.W., Kirkpatrick, W.R., Saville, S., Coco, B.J., White, W., Fothergill, A., Rinaldi, M., Eng, T., Patterson, T.F., and Lopez-Ribot, J. (2003). Multiple patterns of resistance to fluconazole in Candida glabrata isolates from a patient with oropharyngeal candidiasis receiving head and neck radiation. Journal of clinical microbiology *41*, 619-622.

Rex, J.H., Pfaller, M.A., Barry, A.L., Nelson, P.W., and Webb, C.D. (1995a). Antifungal susceptibility testing of isolates from a randomized, multicenter trial of fluconazole versus amphotericin B as treatment of nonneutropenic patients with candidemia. NIAID Mycoses Study Group and the Candidemia Study Group. Antimicrobial agents and chemotherapy *39*, 40-44.

Rex, J.H., Pfaller, M.A., Walsh, T.J., Chaturvedi, V., Espinel-Ingroff, A., Ghannoum, M.A., Gosey, L.L., Odds, F.C., Rinaldi, M.G., Sheehan, D.J.*, et al.* (2001). Antifungal susceptibility testing: practical aspects and current challenges. Clinical microbiology reviews *14*, 643-658, table of contents.

Rex, J.H., Rinaldi, M.G., and Pfaller, M.A. (1995b). Resistance of Candida species to fluconazole. Antimicrobial agents and chemotherapy *39*, 1-8.

Richard, G.F., and Dujon, B. (2006). Molecular evolution of minisatellites in hemiascomycetous yeasts. Molecular biology and evolution *23*, 189-202.

Robbins, N., Caplan, T., and Cowen, L.E. (2017). Molecular Evolution of Antifungal Drug Resistance. Annual review of microbiology *71*, 753-775.

Robbins, N., Wright, G.D., and Cowen, L.E. (2016). Antifungal Drugs: The Current Armamentarium and Development of New Agents. Microbiology spectrum *4*.

Rodero, L., Mellado, E., Rodriguez, A.C., Salve, A., Guelfand, L., Cahn, P., Cuenca-Estrella, M., Davel, G., and Rodriguez-Tudela, J.L. (2003). G484S amino acid substitution in lanosterol 14-alpha demethylase (ERG11) is related to fluconazole resistance in a recurrent Cryptococcus neoformans clinical isolate. Antimicrobial agents and chemotherapy *47*, 3653-3656.

Rodrigues, C.F., Silva, S., and Henriques, M. (2014). Candida glabrata: a review of its features and resistance. European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology *33*, 673-688.

Roemer, T., and Krysan, D.J. (2014). Antifungal drug development: challenges, unmet clinical needs, and new approaches. Cold Spring Harbor perspectives in medicine *4*.

Roy, S., and Thompson, D. (2015). Evolution of regulatory networks in Candida glabrata: learning to live with the human host. Fems Yeast Res *15*.

Salazar, S.B., Wang, C., Munsterkotter, M., Okamoto, M., Takahashi-Nakaguchi, A., Chibana, H., Lopes, M.M., Guldener, U., Butler, G., and Mira, N.P. (2018). Comparative genomic and transcriptomic analyses unveil novel features of azole resistance and adaptation to the human host in Candida glabrata. Fems Yeast Res *18*.

Sandmann, S., de Graaf, A.O., Karimi, M., van der Reijden, B.A., Hellstrom-Lindberg, E., Jansen, J.H., and Dugas, M. (2017). Evaluating Variant Calling Tools for Non-Matched Next-Generation Sequencing Data. Sci Rep *7*, 43169.

Sanglard, D., Ischer, F., and Bille, J. (2001). Role of ATP-binding-cassette transporter genes in high-frequency acquisition of resistance to azole antifungals in Candida glabrata. Antimicrobial agents and chemotherapy *45*, 1174-1183.

Sanglard, D., Ischer, F., Koymans, L., and Bille, J. (1998). Amino acid substitutions in the cytochrome P-450 lanosterol 14alpha-demethylase (CYP51A1) from azole-resistant Candida albicans clinical isolates contribute to resistance to azole antifungal agents. Antimicrobial agents and chemotherapy *42*, 241-253.

Sanguinetti, M., Posteraro, B., Fiori, B., Ranno, S., Torelli, R., and Fadda, G. (2005). Mechanisms of azole resistance in clinical isolates of Candida glabrata collected during a hospital survey of antifungal resistance. Antimicrobial agents and chemotherapy *49*, 668-679.

Santos, M.A., Gomes, A.C., Santos, M.C., Carreto, L.C., and Moura, G.R. (2011). The genetic code of the fungal CTG clade. Comptes rendus biologies *334*, 607-611.

Sasso, M., Roger, C., and Lachaud, L. (2017). Rapid emergence of FKS mutations in Candida glabrata isolates in a peritoneal candidiasis. Medical mycology case reports *16*, 28-30.

Schmieder, R., and Edwards, R. (2011). Quality control and preprocessing of metagenomic datasets. Bioinformatics *27*, 863-864.

Schubert, S., Barker, K.S., Znaidi, S., Schneider, S., Dierolf, F., Dunkel, N., Aid, M., Boucher, G., Rogers, P.D., Raymond, M.*, et al.* (2011). Regulation of Efflux Pump Expression and Drug Resistance by the Transcription Factors Mrr1, Upc2, and Cap1 in Candida albicans. Antimicrobial agents and chemotherapy *55*, 2212-2223.

Selmecki, A., Forche, A., and Berman, J. (2006). Aneuploidy and isochromosome formation in drug-resistant Candida albicans. Science *313*, 367-370.

Selmecki, A., Gerami-Nejad, M., Paulson, C., Forche, A., and Berman, J. (2008). An isochromosome confers drug resistance in vivo by amplification of two genes, ERG11 and TAC1. Molecular microbiology *68*, 624-641.

Seneviratne, C.J., Wang, Y., Jin, L.J., Abiko, Y., and Samaranayake, L.P. (2010). Proteomics of drug resistance in Candida glabrata biofilms. Proteomics *10*, 1444-1454.

Shapiro, R.S., Robbins, N., and Cowen, L.E. (2011). Regulatory circuitry governing fungal development, drug resistance, and disease. Microbiology and molecular biology reviews : MMBR *75*, 213-267.

Singh-Babak, S.D., Babak, T., Diezmann, S., Hill, J.A., Xie, J.L., Chen, Y.L., Poutanen, S.M., Rennie, R.P., Heitman, J., and Cowen, L.E. (2012). Global analysis of the evolution and mechanism of echinocandin resistance in Candida glabrata. PLoS pathogens *8*, e1002718.

Spasskaya, D.S., Karpov, D.S., Mironov, A.S., and Karpov, V.L. (2014). Transcription factor Rpn4 promotes a complex antistress response in Saccharomyces cerevisiae cells exposed to methyl methanesulfonate. Mol Biol+ *48*, 141-149.

Strollo, S., Lionakis, M.S., Adjemian, J., Steiner, C.A., and Prevots, D.R. (2016). Epidemiology of Hospitalizations Associated with Invasive Candidiasis, United States, 2002-2012(1). Emerging infectious diseases *23*, 7-13.

Szweda, P., Gucwa, K., Romanowska, E., Dzierzanowska-Fangrat, K., Naumiuk, L., Brillowska-Dabrowska, A., Wojciechowska-Koszko, I., and Milewski, S. (2015). Mechanisms of azole resistance among clinical isolates of Candida glabrata in Poland. J Med Microbiol *64*, 610-619.

Taff, H.T., Nett, J.E., Zarnowski, R., Ross, K.M., Sanchez, H., Cain, M.T., Hamaker, J., Mitchell, A.P., and Andes, D.R. (2012). A Candida biofilm-induced pathway for matrix glucan delivery: implications for drug resistance. PLoS pathogens *8*, e1002848.

Taura, T., Krebber, H., and Silver, P.A. (1998). A member of the Ran-binding protein family, Yrb2p, is involved in nuclear protein export. Proceedings of the National Academy of Sciences of the United States of America *95*, 7427-7432.

Thakur, J.K., Arthanari, H., Yang, F., Pan, S.J., Fan, X., Breger, J., Frueh, D.P., Gulshan, K., Li, D.K., Mylonakis, E.*, et al.* (2008). A nuclear receptor-like pathway regulating multidrug resistance in fungi. Nature *452*, 604-609.

Thierry, A., Bouchier, C., Dujon, B., and Richard, G.F. (2008). Megasatellites: a peculiar class of giant minisatellites in genes involved in cell adhesion and pathogenicity in Candida glabrata. Nucleic acids research *36*, 5970-5982.

Tian, Y., Gao, N., Ni, Q., Mao, Y., Dong, D., Huang, X., Jiang, C., Li, Z., Zhang, L., Wang, X., *et al.* (2018). Sequence modification of the master regulator Pdr1 interferes with its transcriptional autoregulation and confers altered azole resistance in Candida glabrata. Fems Yeast Res *18*.

Timmermans, B., De Las Penas, A., Castano, I., and Van Dijck, P. (2018). Adhesins in Candida glabrata. Journal of fungi *4*.

Tsai, H.F., Krol, A.A., Sarti, K.E., and Bennett, J.E. (2006). Candida glabrata PDR1, a transcriptional regulator of a pleiotropic drug resistance network, mediates azole resistance in clinical isolates and petite mutants. Antimicrobial agents and chemotherapy *50*, 1384-1392.

Tsai, H.F., Sammons, L.R., Zhang, X., Suffis, S.D., Su, Q., Myers, T.G., Marr, K.A., and Bennett, J.E. (2010). Microarray and molecular analyses of the azole resistance mechanism in Candida glabrata oropharyngeal isolates. Antimicrobial agents and chemotherapy *54*, 3308-3317.

Vale-Silva, L., Ischer, F., Leibundgut-Landmann, S., and Sanglard, D. (2013). Gain-of-function mutations in PDR1, a regulator of antifungal drug resistance in Candida glabrata, control adherence to host cells. Infection and immunity *81*, 1709-1720.

Vale-Silva, L.A., Moeckli, B., Torelli, R., Posteraro, B., Sanguinetti, M., and Sanglard, D. (2016). Upregulation of the Adhesin Gene EPA1 Mediated by PDR1 in Candida glabrata Leads to Enhanced Host Colonization. mSphere *1*.

vanden Bossche, H., Marichal, P., Odds, F.C., Le Jeune, L., and Coene, M.C. (1992). Characterization of an azole-resistant Candida glabrata isolate. Antimicrobial agents and chemotherapy *36*, 2602-2610.

Vandeputte, P., Ferrari, S., and Coste, A.T. (2012). Antifungal resistance and new strategies to control fungal infections. International journal of microbiology *2012*, 713687.

Veltman, D.M., and Insall, R.H. (2010). WASP Family Proteins: Their Evolution and Its Physiological Implications. Molecular biology of the cell *21*, 2880-2893.

Vermitsky, J.P., Earhart, K.D., Smith, W.L., Homayouni, R., Edlind, T.D., and Rogers, P.D. (2006). Pdr1 regulates multidrug resistance in Candida glabrata: gene disruption and genome-wide expression studies. Molecular microbiology *61*, 704-722.

Vermitsky, J.P., and Edlind, T.D. (2004). Azole resistance in Candida glabrata: coordinate upregulation of multidrug transporters and evidence for a Pdr1-like transcription factor. Antimicrobial agents and chemotherapy *48*, 3773-3781.

Verstrepen, K.J., and Klis, F.M. (2006). Flocculation, adhesion and biofilm formation in yeasts. Molecular microbiology *60*, 5-15.

Wahl, L.M., and Krakauer, D.C. (2000). Models of experimental evolution: the role of genetic chance and selective necessity. Genetics *156*, 1437-1448.

Wang, X.G., Xu, H.M., Ha, S.W., Ju, D.H., and Xie, Y.M. (2010). Proteasomal Degradation of Rpn4 in Saccharomyces cerevisiae Is Critical for Cell Viability Under Stressed Conditions. Genetics *184*, 335-U339.

Wang, Y., Lo, W.C., and Chou, C.S. (2017). A modeling study of budding yeast colony formation and its relationship to budding pattern and aging. PLoS computational biology *13*, e1005843.

Weig, M., Jansch, L., Gross, U., De Koster, C.G., Klis, F.M., and De Groot, P.W. (2004). Systematic identification in silico of covalently bound cell wall proteins and analysis of protein-polysaccharide linkages of the human pathogen Candida glabrata. Microbiology *150*, 3129-3144.

Whaley, S.G., Berkow, E.L., Rybak, J.M., Nishimoto, A.T., Barker, K.S., and Rogers, P.D. (2016). Azole Antifungal Resistance in Candida albicans and Emerging Non-albicans Candida Species. Front Microbiol *7*, 2173.

White, T.C., Marr, K.A., and Bowden, R.A. (1998). Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. Clinical microbiology reviews *11*, 382-402.

Wingard, J.R. (1994). Infections due to resistant Candida species in patients with cancer who are receiving chemotherapy. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America *19 Suppl 1*, S49-53.

Wingard, J.R. (1995). Importance of Candida species other than C. albicans as pathogens in oncology patients. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America *20*, 115-125.

Wingard, J.R., Merz, W.G., Rinaldi, M.G., Miller, C.B., Karp, J.E., and Saral, R. (1993). Association of Torulopsis glabrata infections with fluconazole prophylaxis in neutropenic bone marrow transplant patients. Antimicrobial agents and chemotherapy *37*, 1847-1849.

Winter, D.C., Choe, E.Y., and Li, R. (1999). Genetic dissection of the budding yeast Arp2/3 complex: a comparison of the in vivo and structural roles of individual subunits. Proceedings of the National Academy of Sciences of the United States of America *96*, 7288-7293.

Wisplinghoff, H., Bischoff, T., Tallent, S.M., Seifert, H., Wenzel, R.P., and Edmond, M.B. (2004). Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America *39*, 309-317.

Wisplinghoff, H., Seifert, H., Tallent, S.M., Bischoff, T., Wenzel, R.P., and Edmond, M.B. (2003a). Nosocomial bloodstream infections in pediatric patients in United States hospitals: epidemiology, clinical features and susceptibilities. The Pediatric infectious disease journal *22*, 686-691.

Wisplinghoff, H., Seifert, H., Wenzel, R.P., and Edmond, M.B. (2003b). Current trends in the epidemiology of nosocomial bloodstream infections in patients with hematological malignancies and solid neoplasms in hospitals in the United States. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America *36*, 1103-1110.

Wootton, M., Howe, R.A., Hillman, R., Walsh, T.R., Bennett, P.M., and MacGowan, A.P. (2001). A modified population analysis profile (PAP) method to detect hetero-resistance to vancomycin in Staphylococcus aureus in a UK hospital. The Journal of antimicrobial chemotherapy *47*, 399-403.

Yoshikawa, K., Tanaka, T., Ida, Y., Furusawa, C., Hirasawa, T., and Shimizu, H. (2011). Comprehensive phenotypic analysis of single-gene deletion and overexpression strains of Saccharomyces cerevisiae. Yeast *28*, 349-361.

Zeyl, C. (2000). Budding yeast as a model organism for population genetics. Yeast *16*, 773-784.