Universidade do Minho
Escola de Engenharia

Elza Sofia da Silva da Fonseca

Resistance Mechanisms of *Candida glabrata* Biofilms to Fluconazole

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Mestrado em Bioengenharia

Trabalho efetuado sob a orientação da
Doutora Mariana Henriques
e co-orientação da
Doutora Sónia Silva

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DECLARAÇÃO

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Orientador: Doutora Mariana Henriques

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É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA DISSERTAÇÃO APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE;

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Abstract

Candida glabrata has emerged as the second most prevalent fungal pathogen and its ability to form biofilms has been considered one of the most important virulence factors, since biofilms present a high tolerance to antifungal agents used in fungal infection treatment. The mechanisms of biofilm tolerance to antifungal agents remain poorly understood. Thus, the aim of this study was to evaluate the effects of fluconazole (FLU) in the formation and control of *C. glabrata* biofilms, its relation with the expression of genes encoding for ABC transporters, *CDR1*, *SNQ2*, and *PDR1* and how the ergosterol biosynthesis may be affected.

Additionally to the high amounts of proteins and carbohydrates detected in the extracellular matrices in the presence of FLU, this work showed that the overexpression of efflux pumps is a possible mechanism of biofilm tolerance to FLU and this phenomenon alters the structure of *C. glabrata* biofilms by creating cell clusters.

Resumo

Candida glabrata emergiu como o segundo fungo patogénico mais prevalente e a sua capacidade para formar biofilmes tem sido considerado um dos fatores de virulência mais importante, uma vez que os biofilmes apresentam elevada tolerância a agentes antifúngicos usados no tratamento de infecções fúngicas. Os mecanismos de tolerância dos biofilmes continuam por explorar. Por isso, o objetivo deste estudo é avaliar os efeitos do fluconazol (FLU) na formação e controlo dos biofilmes de *C. glabrata*, a sua relação com a expressão de genes que encodam os transportadores ABC, *CDR1*, *SNQ2* e *PDR1* e como a biossíntese do ergosterol pode ser afetada.

Adicionalmente, para as grandes quantidades de proteínas e hidratos de carbono detetados nas matrizes extracelulares, na presença de FLU, este trabalho demonstrou que a sobre-expressão de bombas de efluxo é um possível mecanismo de tolerância dos biofilmes contra o FLU e este fenómeno altera a estrutura dos biofilmes de C*. glabrata* pela criação de agregados de células.

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Nomenclature

ACT-Actin

ANOVA - Analysis of variance

ATCC - American Type Culture Collection

cDNA - complementary Deoxyribonucleic Acid

CFU - Colony Forming Units

CHROMagar - Chromogenic media agar

CLSM - Confocal Laser Scanning Microscopy

CV - Crystal violet

DNA - Deoxyribonucleic Acid

dNTP - desoxynucleoside triphospahte

FLU - Fluconazole

h - Hour

Log - Logarithm

mRNA - messenger Ribonucleic Acid

NCAC - non-*Candida albicans Candida*

PBS - Phosphate Buffer Saline

RT- PCR – Real-time Polymerase Chain Reaction

RNA - Ribonucleic Acid

rpm - rotation per minute

rRNA - ribosomal Ribonucleic Acid

SDA - Sabouraud dextrose agar

SDB - Sabouraud dextrose broth

SD - Standard deviation

SEM - Scanning Electron Microscopy

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Chapter I

Background and Objectives

I.1. Background

Most cases of candidiasis have been attributed to *Candida albicans*, but recently, non-*Candida albicans Candida* (NCAC) species, as *Candida glabrata*, have been identified as common pathogens. The incidence of systemic infections caused by *C. glabrata* increased dramatically throughout the 1990s and became the most common cause of candidiasis after *C. albicans*. *Candida glabrata* systemic infections are a subject of considerable concern due to the tendency of this species to rapidly develop resistance to azole antifungal agents, especially fluconazole, and polyenes like amphotericin B. Moreover, this species is also important due to the high mortality rates associated with *C. glabrata* fungemia. Adherence to host surfaces including medical devices, secretion of hydrolytic enzymes and specially biofilm formation are virulence factors that are associated with *Candida* pathogenicity.

Biofilms formed by *Candida* isolates have been associated with higher morbidity and mortality rates compared with isolates unable to form biofilms, due to the significant resistance to antifungal therapy conferred by the complex biofilm structure and composition. Despite, the lack of knowledge about the exact mechanism of biofilm resistance to antifungals, it is believed that this is a complex multifactorial phenomenon. Actually, restricted penetration of drugs through the biofilm matrix, phenotypic changes resulting from a decreased growth rate or nutrient limitation, expression of resistance genes induced by contact with a surface and the presence of a small number of "persister" cells are hypothesized as mechanisms of biofilm resistance.

At the present, little is known about *C. glabrata* biofilms resistance, so the aim of this project is to study the resistance mechanisms of *C. glabrata* biofilms to antifungal agents. It is expected that these studies will ultimately contribute towards the identification of targets for novel therapeutics against *C. glabrata* infections.

I.2. Objective

The comprehension of resistance mechanisms of *C. glabrata* biofilms is the key to succeed on the *Candida* infections treatment, since the biofilms present much higher MICs for the common antifungal agents used, which in most cases, results on treatment failure. Thus, the main goal of this project is to study the resistance mechanisms of *C. glabrata* biofilms to fluconazole. To achieve this goal, the effects of FLU in the formation and control of *C. glabrata* biofilms, in the extracellular matrix composition was evaluated, as well as on ABC transporter genes expression (*CDR1*, *SNQ2*, *PDR1*) and on ERG genes (*ERG1*, *ERG3*, *ERG6*, *ERG9*, *ERG11*) that are involved in the ergosterol biosynthesis.

Therefore, under the subject of this project it is expected to provide more knowledge for the development of new and more specific therapies for *Candida* infections, thus rising patients health and lowering the costs involved in the wrong application of these agents.

I.3. Structure of the thesis

This dissertation is divided in six different chapters in order to present the work done during the time of investigation:

I. Background and objectives

This chapter will present the context and the objectives that gave wings to this thesis.

II. Introduction

This chapter will be focused in the theoretical basis associated to this work.

III. Materials and methodology

In this chapter the materials and the methods and techniques used in the experiments will be presented.

IV. Analysis of the results

In this chapter all results obtained during the whole experimental work will be included.

V. Discussion of the results

In this chapter the discussion of all the results obtained will be exposed.

VI. Conclusions

In this chapter the main conclusions obtained from the realized work will be presented and some works will be suggested to the future.

VII. References

The all bibliography used to the comprehension, execution and written of this dissertation will be listed in this chapter.

The work presented in this thesis was developed in the Center of Biological Engineering, Department of Biological Engineering of the University of Minho.

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Chapter II

Introduction

II.1. *Candida* Discovery

"Thrush" was documented for the first time by Pepys in 1665. However, only in 1846, a scientific approach to the study of thrush, carry out by Berg, showed the presence and relationship of the fungus with the disease. After these studies, the idea that the organism could cause several forms of the same disease became clear. In 1792 and then in 1849, Frank and Wilkinson, respectively, observed that aphthae occurs not only in oral cavity but also in sexual organs and that a dimorphic fungus was the probable cause (Calderone 2002).

Even so, the identity of the organism that causes the disease was only approved in 1954. Langenbeck was the first one that observed the fungus, in 1839, but the identity of the organism was incorrect. Then, in 1842, Gruby studied Langenbeck's organism and concluded that it was a species of *Sporotrichum*. Five years late, Robin reclassified it as *Oidium albicans* (Calderone 2002)

It was only in 1923, that the generic name *Candida* was proposed to the organism responsible for thrush, when Berkhout proved that it was not a species of *Monilia*, but a fungus that grows in plant materials and clearly morphologically different from *Candida*, which was associated until that time. The whitish colonies on agar or the oral lesion of aphthae or thrush was probably the reason for the name *Candida*, which derived from the Latin phrase *toga candida*, which was used to describe a special white robe worn by candidates for the Roma Senate (Calderone 2002).

In the past 50 years, the number of new species of *Candida* described increased to approximately 150 (Calderone 2002).

II.2. *Candida* Characteristics

Candida species are ubiquitous organisms and most of them are not human pathogens. The genus *Candida* is very heterogeneous and its principal characteristics will be described below.

II.2.1. Cell Biology

The genus *Candida* is a genus consisting of yeasts that do not possess a known natural sexual cycle. Most *Candida* species exist as spherical to ovoid budding yeast cells or blastospores, typically 4 to 6 μm in diameter. Some *Candida* species are capable of producing chains of elongated blastospores termed pseudohyphae both *in vivo* and under certain conditions *in vitro*. Table II.1 summarizes these properties, for some of the most relevant species (Calderone 2002).

Table II. 1. Morphological characteristics of *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis* and *Candida glabrata* species. Adapted from (Silva, Negri et al. 2012).

Species	Germ tube	Pseudohyphae	Yeast size (μm)	CHROM-agar colony colour
C. albicans	+	+	4-6 x 6-10	Blue-green
C. tropicalis	-	+	4-8 x 5-11	Dark blue
C. glabrata	-	-	1-4	White, Pink-purple
C. parapsilosis	-	+	2.5-4 x 2.5-9	White

The cell wall of pathogens is critical to their interaction with host cells. For pathogenic fungi the cell wall represents the primary way in which the organism interacts with its host.

Yeast and hyphal cell walls are similar qualitatively but different quantitatively in specific components, as chitin. Thus, the mycelial cell wall exhibited four- to fivefold higher level of chitin (Calderone 2002).

The cell wall of *Candida* is approximately 80 to 90% carbohydrate and β-glucan (branched polymers of glucose), mannan (polymers of mannose) and chitin (polymers of β-1,4 N-acetyl-D) are the primary constituents. The most abundant component of *Candida* cell wall is β-glucan (β-1,3 and β-1,6 glucose polymer) that account fot 47 to 60% of the weight of the cell wall, followed by mannoproteins that account for approximately 40% of the total cell wall polysaccharides, chitin that account for 0.5 to

3% and glycolipids that account for 1 to 7% by dry weight of the cell wall. Ultrastructural and biochemical observation revealed a layer arrangement: an inner wall composed of structural polysaccharides and an outer layer containing primarily mannan, mannoproteins or nonglycosylated proteins (Chauhan, Li et al. 2002).

The biomolecules of the cell wall that are not found in mammalian cells are potential targets for the identification of antifungal agents.

II.2.2. Growth Forms of *Candida*

The genus *Candida* is composed of an extremely heterogeneous group of organisms that grow as yeast, but most members of the genus also produce a filamentous type of growth (pseudohyphae, pseudomycelium). However, *C. albicans* and *C. dubliniensis* form true hyphae in addition to pseudohyphae. Thus, both species are considered polymorphic (Calderone 2002).

Pseudohyphae are formed from yeast cells or hyphae by budding, but the new cell remains attached to the parent one and elongates, resulting in filaments with constrictions at the cell-cell junctions of the filaments (Calderone 2002).

True hyphae are formed from yeast cells or as branches of existing hyphae. Outgrowths of the yeast cells (germ tubes) grow by apical extension and cross walls (septa) are formed behind the growing tip of the hyphae. Budding occurs laterally just behind the septa, the latter of which are perpendicular to the main axis of the hyphae (Calderone 2002).

The pseudohyphae appear to be an intermediate growth form of yeast and hyphal morphologies (Calderone 2002).

Germination can be induced in complex media, chemically defined media and serum. Temperatures greater than 35 ^{o}C, pH of 6.5 to 7.0 or slightly alkaline and inoculum of $<10^{6}$ mL^{-1} favor germination, whereas glucose as a sole carbon source, lower temperatures and an acid pH favor yeast growth (Calderone 2002).

II.2.3. Virulence

Candida is a sophisticated pathogen. Although there are about 150 species of *Candida*, approximately 65% of *Candida* species are unable to grow at a temperature of 37 ^{o}C, a prerequisite for an organism to be a successful pathogen (Calderone and Gow 2002). There are several known virulence factors contributing to *Candida* pathogenicity that include adherence to epithelial and endothelial cells, proteinase production, hyphae

and pseudohyphae formation, phenotypic switching, phospholipase production and antigenic modulation as a result of pseudohyphae formation (Fidel, Vazquez et al. 1999).

Adhesion is one of the most important virulence factors of *Candida* species. Adhesins are cell-surface components of *Candida* that promote host recognition and colonization (Calderone and Gow 2002).

Most of the medically important species within the genus *Candida* possess the ability to produce pseudomycelium and are otherwise morphologically very similar. The interconversion of yeast forms to filamentous growth, a process named by morphogenesis, is associated with invasiveness of the organism and also contributes for virulence (Calderone and Gow 2002).

The secretion of digestive enzymes such as the SAPs, a family of secreted aspartyl proteinases, and phospholipase B (PLB), is also required for their virulence since these hydrolytic enzymes can degrade host tissues and thus contribute for their invasion (Calderone and Gow 2002).

As opportunistic pathogens, *Candida* species can invade every tissue of the human body, depending on the integrity of the host immune system. Its capacity to live both as a commensal and pathogen, to evade the immune system, to overcome drug therapy, to invade a variety of body location and to adjust so rapidly to changes in host physiology suggests that it has extraordinary phenotypic plasticity and can adapt rapidly to environmental changes. "High-frequency phenotypic switching" can generate a variety of general phenotypes, occurs spontaneously, moreover can be affected by environmental changes and can have a profound effect on pathogenic traits. Switching is regulated by a number of phase-specific genes in a combinatorial fashion and a high proportion of these genes directly or indirectly have impact on pathogenesis and virulence (Soll 2002).

The development of biofilms, the most prevalent growth form of microorganisms, is usually observed after initial attachment of *Candida* to host or/and medical devices. Biofilms are described as surface-associated communities of microorganisms embedded within an extracellular matrix and are an important virulence factors for a number of *Candida* species, as they confer significant resistance to antifungal therapy by limiting the penetration of substances through the matrix and protecting cells from host immune responses. Furthermore, biofilms formed by *C. albicans*, *C. parapsilosis*, *C. tropicalis* and *C. glabrata* isolates have been associated

with higher morbidity and mortality rates compared with isolates unable to form biofilms (Silva, Negri et al. 2012).

II.3. Non-*Candida albicans Candida* species

Candidiasis remains an important clinical problem, primarily in the immunocompromised patient population. *Candida albicans* initially was the most important pathogen but now non-*Candida albicans Candida* (NCAC) species, as *Candida tropicalis, Candida parapsilosis, Candida glabrata, Candida krusei* and C*andida dubliniesis,* have gained clinical importance (Moran, Sullivan et al. 2002; Silva, Negri et al. 2011).

The apparent increased emergence of NCAC species in human candidiasis may be related to improvements in diagnostic methods, such as the use of chromogenic media with the ability to differentiate *Candida* species, as well as the introduction of molecular techniques in the routine diagnosis of fungemia. Nevertheless, the high prevalence of NCAC species in infections could also be a reflection of their inherent higher level of resistance to certain antifungal drugs compared to *C. albicans*, as this would promote their persistence in mixed species infections treated with traditional antifungal agents (Silva, Negri et al. 2012).

NCAC species are a very heterogeneous group of organisms that are fundamentally different from each other and from *C. albicans* at the biological level (Table I). The virulence of different NCAC species in human and in animal models of infection varies considerably, for example, the ability or the inability to form pseudohyphae, the family of adhesins, the kind of hydrolytic enzymes produced (Moran, Sullivan et al. 2002).

In the NCAC species, *C. glabrata* is considered relatively nonpathogenic in animal models, which suggests that it has few virulence attributes. However, high mortality rate has been associated to this *Candida* (Fidel, Vazquez et al. 1999). Few studies had been conducted on virulence of *C. glabrata*, that's why great importance is given to this *Candida* in this work.

II.4. *Candida glabrata*

Historically, *C. glabrata* was considered a relatively nonpathogenic saprophyte of the normal flora of healthy individuals, rarely causing serious infection in humans (Fidel, Vazquez et al. 1999). However, the incidence of systemic infections caused by *C. glabrata* increased dramatically throughout the 1990s and depending on the site of infection *C. glabrata* is often the second or third most common cause of candidiasis after *C. albicans* and is also the NCAC species most commonly recovered from the oral cavities of HIV-infected individuals (Fidel, Vazquez et al. 1999; Moran, Sullivan et al. 2002).

Candida glabrata systemic infections are a subject of considerable concern due to the tendency of this species to rapidly develop resistance to azole antifungal agents and due to the high mortality rate associated with *C. glabrata* fungemia (Moran, Sullivan et al. 2002).

II.4.1. Epidemiology

Data from the 90s show that approximately 31 to 55% of the oral cavity of healthy individuals is colonized by *Candida* species and this colonization increases with severity of illness and duration of hospitalization. Initially, *C. albicans* accounted for 70 to 80% of the isolates recovered from infected patients, *C. glabrata* and *C. tropicalis* each accounted for approximately 5 to 8% of isolates, while other NCAC species occur only rarely. However, a change in epidemiology was observed. Although *C. albicans* is the most common fungal species isolated from blood, *C. glabrata* started to appear associated with an equally high mortality rate. The incidence of *C. glabrata* is higher in adults than in children and lower in neonates and, despite had being considered a relatively nonpathogenic saprophyte of the normal flora of healthy individuals and certainly not readily associated with serious infection in humans, it is of special importance because of its innately increased resistance to antifungal agents, specifically the azoles (Fidel, Vazquez et al. 1999; Hachem, Hanna et al. 2008; Silva, Negri et al. 2012).

More recently, in the United States, a study demonstrated that *C. glabrata* has increased as a cause of invasive candidiasis from 18% of all blood stream infection

isolates in the time period of 1992-2001 to 25% in 2001- 2007 with a concomitant increase in fluconazole resistance from 9% to 14%. Another recent study demonstrated that resistance to both azoles and echinocandins was most prominent among isolates of *C. glabrata* with the highest resistance rates to echinocandins (16.7%), fluconazole (16.7%), posaconazole (5.0%) and voriconazole (11.0%) among isolates from the 20-39-year age group (Pfaller 2012).

The emergence of multidrug resistant (MDR) in *C. glabrata* is a real fear since that neither azoles nor amphotericin B are an optimal approach for therapy for *C. glabrata* infection (Pfaller 2012). For this reason, future surveillance efforts should focus on emergence of these potentially MDR strains of *C. glabrata* and the knowledge of the resistance mechanisms to antifungal agents should be a priority.

II.4.2. Cell Biology

Candida glabrata is a nondimorphic yeast that exists as small blastoconidia (1 to 4 μm) under some environmental conditions as a pathogen. In fact, *C. glabrata* is the only *Candida* species that does not form pseudohyphae at temperatures above 37 °C (Table II.1) (Fidel, Vazquez et al. 1999; Calderone 2002).

On Sabouraud dextrose agar, *C. glabrata* forms glistening, smooth, cream-colored colonies, which are relatively indistinguishable from those of other *Candida* species except for their relative size, which is quite small. On Chromagar, a differential medium that distinguishes *Candida* species by color as a result of biochemical reactions, *C. glabrata* colonies appear pink to purple, in contrast to *C. albicans* colonies, which appear green to blue-green (Table II.1) (Fidel, Vazquez et al. 1999; Calderone 2002). Among the critical distinguishing characteristics of *C. glabrata* are its haploid genome, in contrast to the diploid genome of *C. albicans* and several other NCAC species and its small-subunit rRNA (Fidel, Vazquez et al. 1999; Ernst and Bockmuhl 2002).

The biochemical reactions of *C. glabrata* are also quite distinct. *Candida glabrata* ferments and assimilates only glucose and trehalose, while *C. albicans* ferments and assimilates a high number of sugars (Fidel, Vazquez et al. 1999; Calderone 2002).

II.4.3. Virulence

Candida glabrata is considered less pathogenic than *C. albicans* and other NCAC species, particularly in animal models of infection, although it is being associated with virulent infection in several immunocompromised individuals. *C. glabrata* adheres poorly to host surfaces and produces less proteinases than *C. tropicalis* and *C. parapsilosis*. Adhesins, cell surface proteins that are involved in specific adherence, encoded by *EPA* gene family are major group of adhesins in *C. glabrata* and it is known that EPA1p is a calcium-dependent lectin (Fidel, Vazquez et al. 1999; Moran, Sullivan et al. 2002; Silva, Negri et al. 2011; Silva, Negri et al. 2012).

Haemolysins are considered key virulence factors since they enable pathogen grow in the host using haemin or haemoglobin as a source of iron. Luo (Silva, Negri et al. 2012) observed that *C. glabrata* is able to produce haemolysins *in vitro*, inducing partial or total erythrocyte lyses and showed that a haemolysinlike protein (HLP) gene was associated with the haemolytic activity of *C. glabrata*. But other authors only observed production of haemolysins by *C. albicans* (Silva, Negri et al. 2012).

It is known that *C. glabrata* is unable to produce filamentous forms (hyphae or pseudohyphae) *in vivo* an important virulence factor required for tissue invasion (Fidel, Vazquez et al. 1999; Moran, Sullivan et al. 2002).

Switching was firstly reported in *C. albicans*, but it has been demonstrated in other *Candida* species such as *C. glabrata*, *C. tropicalis* and *C. parapsilosis*. Using an indicator agar 1 mM $CuSO_4$, reversible switching in *C. glabrata* is demonstrated at high frequency between a white to light brown and dark brown colony phenotype. As in *C. albicans*, switching in *C. glabrata* is accompanied by the differential expression of genes: MT-II metallothionein gene and HLP gene that encodes a hemolysis-like protein. These genes are expressed in a graded fashion that correlates with the intensity of pigmentation. One of the genes is involved in copper detoxification while the other may be involved in red blood cell lysis which suggests that just as in the case of *C. albicans*, switching in *C. glabrata* is pleiotropic and again may represent a high order of virulence trait and play a role in causing symptomatic infections (Fidel, Vazquez et al. 1999; Soll 2002).

Biofilm formation is another virulence factor of *C. glabrata*, since biofilms limit the penetration of substances through the matrix and protect cells from host immune responses. The formation of mature biofilms and subsequent production of extracellular

matrix is strongly dependent on species, strain and environmental conditions (pH, medium composition, oxygen) and, in the case of *C. glabrata*, it was recently showed that a higher biofilm biomass is produced on silicone surfaces in the presence of urine, compared to *C. parapsilosis* and *C. tropicalis*. The opposite was found for biofilms formed in Sabouraud dextrose broth. Thus, biofilm formation by *C. glabrata* is lower compared with other NCAC species, when grown in rich culture media. *Candida albicans* biofilm matrix is mainly composed of carbohydrates, proteins, phosphorus and hexosamines. However, Silva (Silva, Negri et al. 2012) reported that the extracellular matrix of *C. glabrata* biofilm is characterized by a high level of both proteins and carbohydrates, while the matrix of *C. parapsilosis* biofilm is mostly composed by carbohydrates and the matrix of *C. tropicallis* biofilm exhibits low levels of both proteins and carbohydrates (Silva, Negri et al. 2012).

II.5. Mechanisms of Resistance to Antifungal Agents

Both the frequency of invasive fungal infections (IFIs) and the resistance to antifungal therapy continue to increase, despite the introduction of new antifungal agents. *In vitro* susceptibility testing is often used to study resistance/sensibility to specific agents against microorganisms. Standardized methods for reliable *in vitro* antifungal susceptibility testing are now available from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) in Europe. Epidemiologic surveys that examine local and regional data can be used to develop empiric treatment strategies and are essential in tracking resistance trends (Pfaller 2012).

Various mechanisms can lead to the acquired resistance of *Candida* species to antifungal agents, like the induction of the efflux pumps encoded by the *MDR* or *CDR* genes and the acquisition of point mutations in the genes encoding for the targeted enzymes (Pfaller 2012). Moreover, it has been reported that biofilm formation confers significant resistance to the antifungal therapies (Baillie and Douglas 2000).

Antifungal resistance is associated with elevated minimum inhibitory concentrations, poorer clinical outcomes, and breakthrough infections during antifungal treatment and prophylaxis (Pfaller 2012).

II.5.1. Antifungal Agents and Resistance

The identification of antifungal drugs began in the late 1940s and continues today.

Antifungal treatments against *Candida* infections are hampered by several factors including the limited number of active agents, the emergence of refractory fungal species and the development of resistance. This situation has triggered the search for new antifungal agents with novel modes of action. Different cellular processes involved in the biosynthesis of components required for the growth of fungal cells have been targeted by antifungal agents (Sanglard and Bille 2002). Actually, the classification of these antifungal agents is based on their target of activity (Figure II.1).

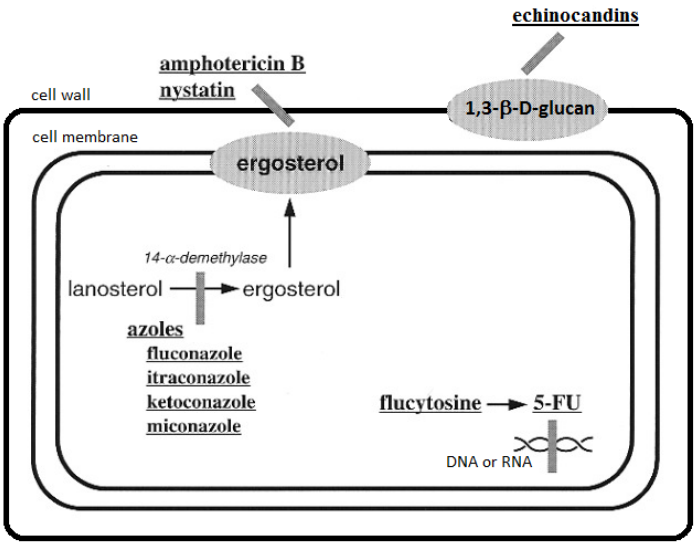

Figure II. 1. Mechanisms of action of antifungal agents: target molecules.

Ergosterol biosynthesis is specific to fungi and is necessary for their growth and this feature has been largely exploited for the design and isolation of antifungal agents such as polyenes and azoles (Sanglard and Bille 2002). Moreover, components of the fungi cell wall are also targets for some antifungal agents as for example echinocandins (Silva, Negri et al. 2012). 5-fluorocytosine (5-FC) is another drug currently used against *Candida*, which can be incorporated into RNA molecules and subsequently interferes with the synthesis of proteins (Sanglard and Bille 2002) (Figure II.1).

Antifungal resistance can be defined as *in vitro* or clinical resistance. *In vitro* resistance can be subdivided into primary resistance and secondary resistance. Primary resistance (intrinsic or innate resistance) occurs when the organism is naturally resistant to the antifungal agent (e.g., *C. krusei*, which is known to be universally resistant to fluconazole). Secondary resistance (acquired resistance) is said to occur when the infecting organism or pathogen becomes resistant to the antifungal agent, in others words, its growth is inhibited by an antimicrobial agent concentration higher than the range seen for wild-type strains (Fidel, Vazquez et al. 1999). Clinical resistance is defined by the situation in which the infecting organism is inhibited by an antimicrobial concentration that is higher than could be safely achieved with normal dosing (Pfaller 2012).

Table II.2 presents the Minimum Inhibitory Concentrations (MICs) of amphotericin B, fluoconazole and voriconazole in *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis* and *C. tropicalis*, data collected from EUCAST.

Table II. 2. Minimum Inhibitory Concentration (MIC) of amphotericin B, fluoconazole and voriconazole (mg/L) in *Candida spp*.

	C. albicans	*C. glabrata*	*C. krusei*	*C. parapsilosis*	*C. tropicalis*
Amphotericin B	0.032 – 1	0.032 – 2	0.12 – 2	0.032 – 2	0.032 – 2
Fluoconazole	0.12 – 128	2 – 128	8 – 128	0.12 – 8	0.12 – 128
Voriconazole	0.004 – 0.25	0.016 – 4	0.016 – 2	0.008 – 0.12	0.008 – 16

The action of antifungal agents (Figure 1) and the mechanisms of resistance against these antifungal agents in *C. glabrata* are described in the sections below.

II.5.1.1. Polyenes

In 1950, Hazen and Brown (Sanglard and Bille 2002) identified the first antifungal agent, a polyene called nystatin. Then, other polyene antifungal agents, as amphotericin B (AmB) (Figure II.2), were isolated by Vandeputte and Gold from *Streptomyces nodosus*. AmB can form soluble salts in both basic and acidic environments, is not orally nor intramuscularly absorbed, and is virtually insoluble in water. Systemic and renal problems are often encountered with AmB and to reduce its unwanted side effects, AmB has been formulated in liposomes, lipid complexes and

colloidal suspensions to allow the use of higher doses of AmB and reduce its toxic effects to mammalian cells.

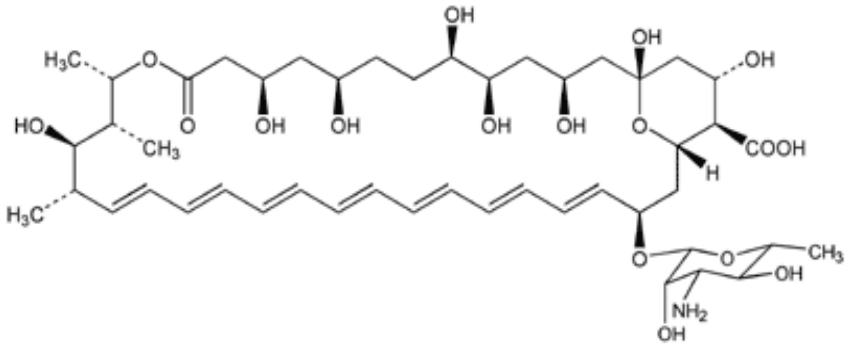

Figure II. 2 Structure of amphotericin B (AmB). Adapted from (Doctor Fungus 2010 [http://www.doctorfungus.org/thedrugs/Ampho_Deoxycholate.htm]

Polyenes bind to ergosterol (Figure II.3) in the bilayer membrane of susceptible fungi. Aqueous pores result from the interaction of polyene molecules linked to the membrane sterols, leading to altered permeability, leakage of vital cytoplasmic components and death of the organism. Polyenes can also bind to cholesterol, which accounts for much of their human toxicity. However, AmB has much higher affinity for ergosterol than for cholesterol (Sanglard and Bille 2002).

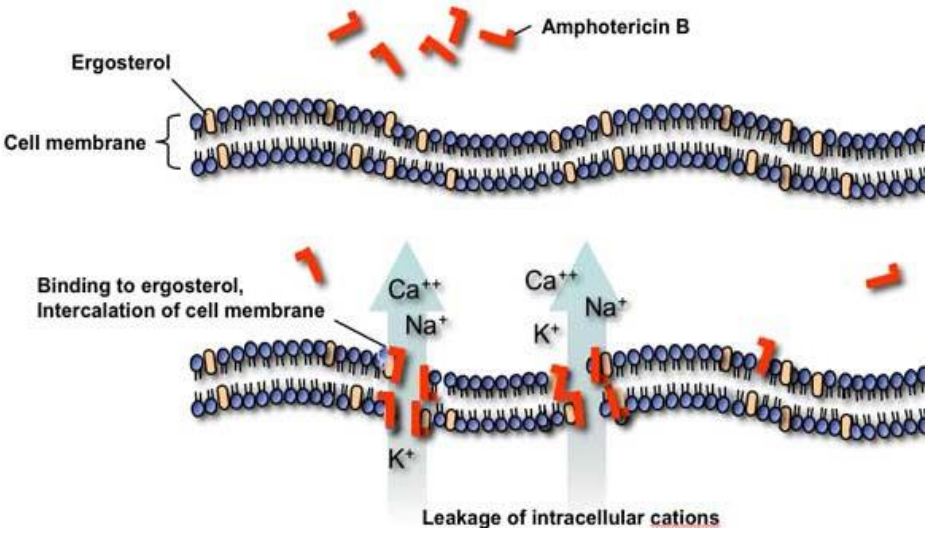

Figure II. 3. Mechanism of action of amphotericin B. Adapted from Doctor Fungus 2010 [http://www.doctorfungus.org/thedrugs/antif_pharm.htm].

One of the mechanisms of resistance to polyenes is believed to result from the alteration of sterol content or composition in the cell membrane. It has been described that among *Candida* species, polyene resistance was usually due to defective ergosterol biosynthesis and most likely resulted from mutation in the *ERG3* gene that produces altered δ5,6-sterol desaturase activity. Mutation in *ERG11* (gene encoding for lanosterol 14α-demethylase, required for sterol biosynthesis) and in *ERG6* (a gene that is required for normal membrane function, but is not essential for sterol biosynthesis) may generate polyene resistance. In *C. glabrata* isolates mutations in the *ERG6* gene were observed (Silva, Negri et al. 2012).

II.5.1.2. 5-fluorocytosine

5-fluorocytosine (5-FC) belongs to the class of pyrimidine analogs that was developed in the 1950s as a potential antineoplastic agent. It is highly water soluble so it can be administrated by oral or intravenous routes (via) (Sanglard and Bille 2002).

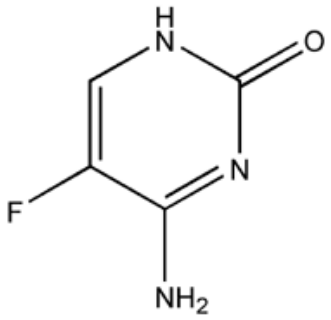

Figure II. 4. Structure of 5-fluorocytosine (5-FC). Adapted from Doctor Fungus 2010 [http://www.doctorfungus.org/thedrugs/Flucytosine.htm].

5-FC is taken up by fungal cells (Figure II.5) by a cytosine permease and is deaminated by a cytosine deaminase to 5-fluorouracil (5-FU). 5-FU is a potent antimetabolite that can be converted to a nucleoside triphosphate and when incorporated into RNA causes miscoding. In other hand, 5-FU can be converted to a deoxynucleoside which inhibits thymidylate synthase and thereby, DNA synthesis. 5-FC has low toxicity in mammalian cells, since cytosine deaminase is absent or poorly active in these cells. However, the conversion of 5-FC to 5-FU is possible by intestinal bacteria and therefore 5-FC can show toxicity in oral formulation and 5-FU, despite being a potent anticancer agent, it is impermeable to fungal cells (Sanglard and Bille 2002).

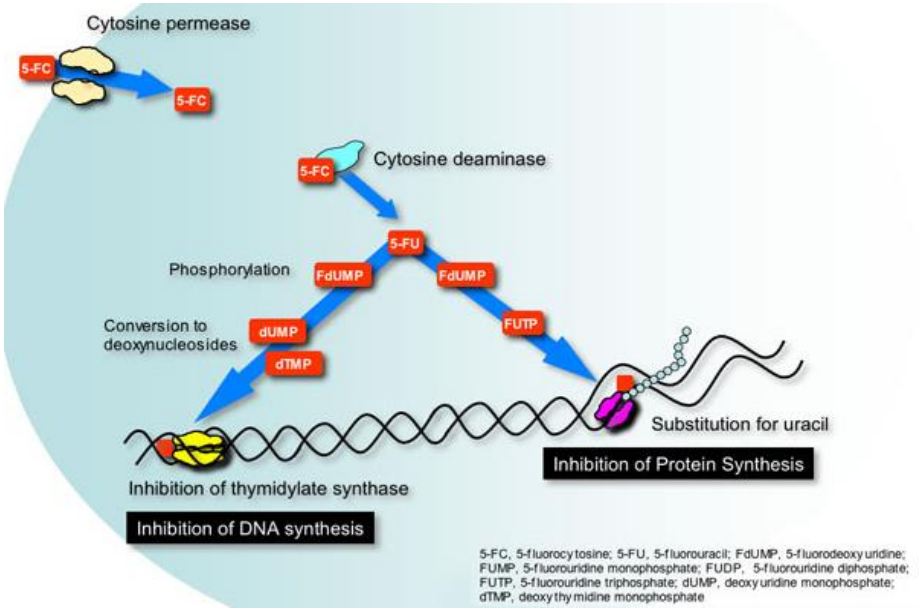

Figure II. 5. Mechanism of action of 5-fluorocytosine. Adapted from Doctor Fungus 2010 [http://www.doctorfungus.org/thedrugs/antif_pharm.htm].

Mechanisms of resistance to 5-FC are possible due to the multiple intracellular enzymatic steps required for its action. These include alterations in the target enzymes UMP pyrophosphorylase, cytosine permease and cytosine deaminase, or increased production of pyrimidines. Due to the multiple steps in its mode of action, including transport into the cell and deamination of the active compound, and due to its extremely narrow spectrum of action 5-FC is normally used only in combination with other agents, including amB and fluconazole (Silva, Negri et al. 2012).

II.5.1.3. Azoles

Since pharmaceutical industry attributed great importance to fungal diseases, more drugs have been developed, and the azoles are a good example. Miconazol was the first azole developed against fungus, followed by the discovery of the triazoles such as fluconazole (Figure II.6) and itraconazole that are less toxic than amphotericin B, although being fungistatic (Sanglard and Bille 2002).

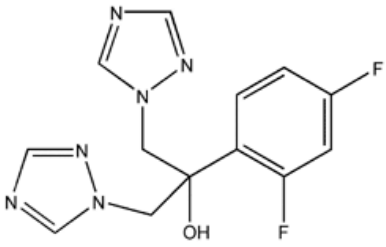

Figure II. 6. Structure of fluconazole (FLU). Adapted from (Doctor Fungus 2010 [http://www.doctorfungus.org/thedrugs/Fluconazole.htm])

Azoles have a cytochrome P450 as a common cellular target in yeast or fungi. Cytochrome P450 is involved in the 14α-demethylation of lanosterol. The unhindered nitrogen of the imidazole or triazole ring of azole antifungal agents binds to the heme iron of the cytochrome P450 as a sixth ligand, thus inhibiting the enzymatic reaction (Figure II.7). As a result ergosterol content in the cell membrane is depleted, membrane structure and functions are altered, and fungal growth is inhibited (Fidel, Vazquez et al. 1999; Sanglard and Bille 2002; Pfaller 2012).

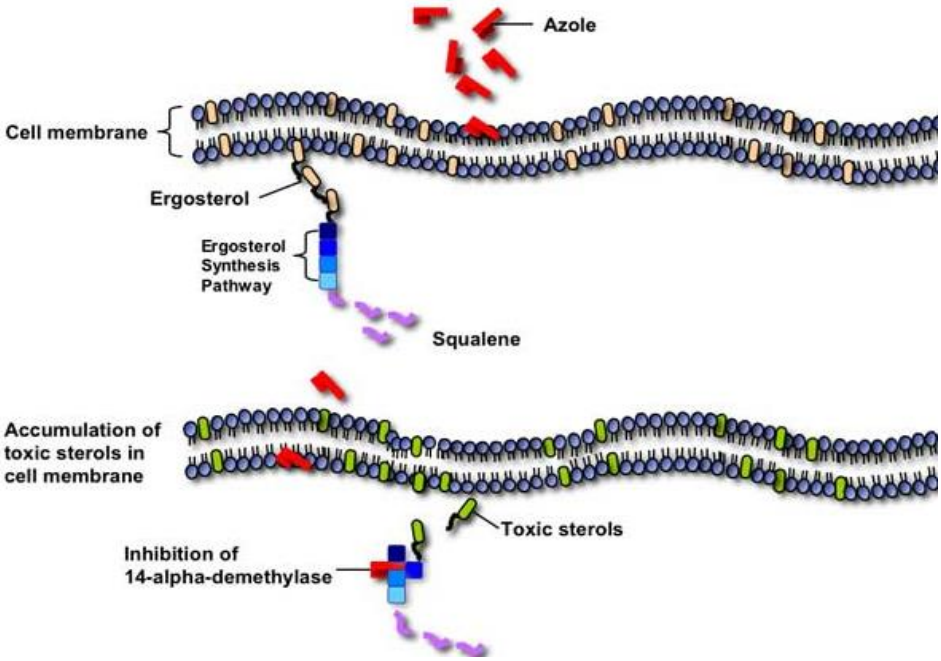

Figure II. 7. Mechanism of action of azoles. Adapted from Doctor Fungus 2010 [http://www.doctorfungus.org/thedrugs/antif_pharm.htm].

There are four principal mechanisms of azole resistance that have been described in *Candida* species. The first mechanism is the induction of efflux pumps that lead to decreased drug concentration at the enzyme target within the fungal cell. In *C. glabrata* the efflux pumps are encoded by *CgCDR1* and *CgCDR2* genes and the up-regulation of these genes has been associated to azole resistance. The second mechanism common in *Candida* species is the acquisition of point mutation in *ERG11*. Thus, an altered enzyme is synthesized with reduced affinity for or incapacity to bind azoles. The third mechanism, which can be associated with the second one, is the overexpression of the altered target enzyme. However, the up-regulation of altered target enzymes does not appear to be a major cause of azole resistance in *Candida*. Finally, the last mechanism

of azole resistance in *Candida* species involves the development of bypass pathways, which negate the membrane-disruptive effects of azole drugs that are associated with inhibited fungal growth. This has been linked with mutation of the *ERG3* gene in certain resistant strains of *Candida*. A study (Pfaller 2012) in *C. albicans* demonstrated an additive nature of resistance mechanisms in *Candida* species for azoles: the control strain with basal expression of *CDR* and WT *ERG11* genes in both alleles, as expected, has low MICs for both fluconazole and voriconazole by comparison with MICs for both azoles in the strain with overexpression of *CDR* and point mutations in both *ERG11* alleles that are much higher. In addition the MICs for fluconazole and voriconazole are approximately twice as high in the strain with basal *CDR* expression and point mutations in both *ERG11* alleles as in the strain with basal *CDR* expression and a point mutation in only one of the *ERG11* alleles.

II.5.1.4. Echinocandins

The first echinocandin isolated was anidulafungin in 1974. Later, in 1989, caspofugin (Figure II.8) was discovered and micafugin was the last to be synthesized in 1990 (Cortés and Russi 2011).

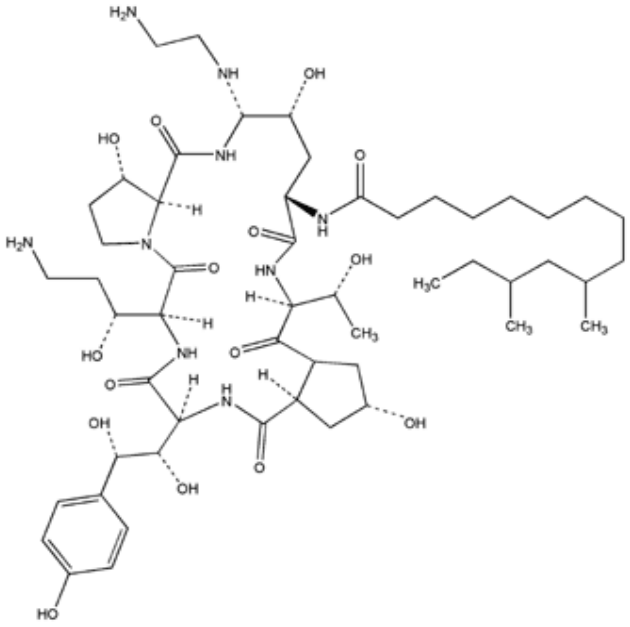

Figure II. 8. Structure of caspofugin. Adapted from Doctor Funfus 2010 [http://www.doctorfungus.org/Thedrugs/Caspofungin.htm].

The echinocandins – anidulafungin, caspofugin and micafungin – are lipopeptides that bind to 1,3-β-D-glucan synthase, enzyme responsible for the biosynthesis of 1,3-β-D-glucan (a component of the cell wall), causing the formation of a defective cell wall associated with cellular instability and lysis in yeasts and aberrant hyphal growth in molds (Figure II.9) (Cortés and Russi 2011; Pfaller 2012).

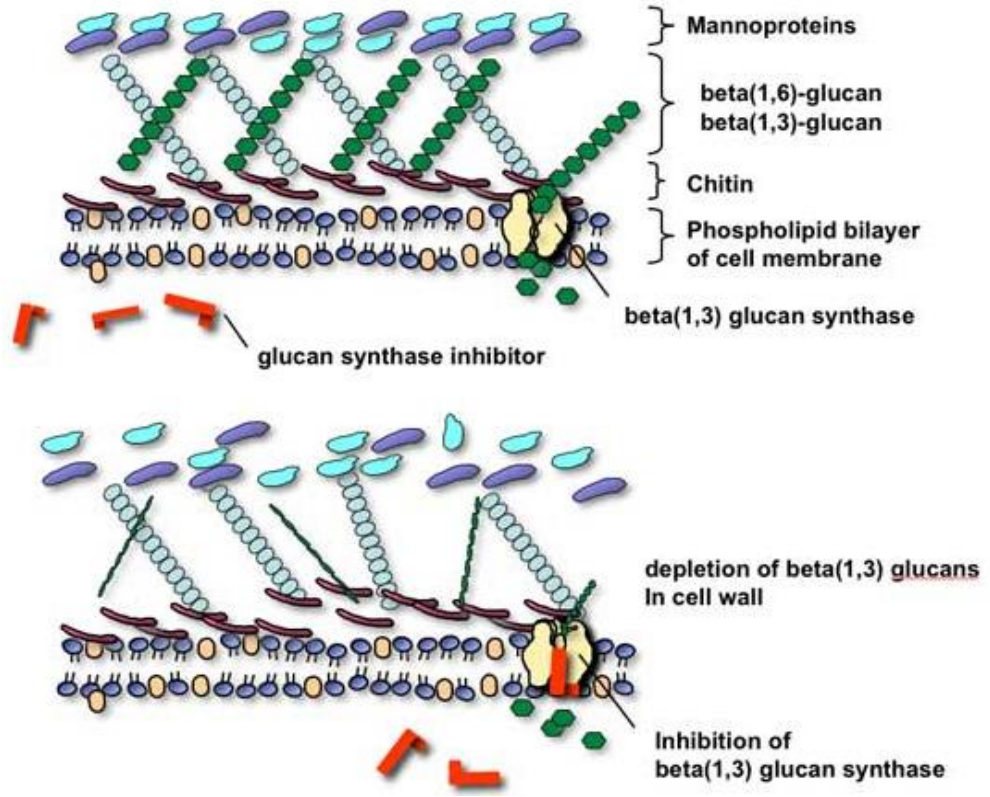

Figure II. 9. Mechanism of action of echinocandins. Adapted from Doctor Fungus 2010 [http://www.doctorfungus.org/thedrugs/antif_pharm.htm].

Reduced susceptibility or resistance of *Candida* to echinocandins has been linked with point mutations in two "hot-spot" regions (HS1 and HS2) of *FKS1*, the gene encoding for the major and presumed catalytic subunit of 1,3-β -D-glucan synthase. This resistance mechanism has been demonstrated in *C. albicans* and NCAC species as *C. glabrata*, *C. krusei*, *C. tropicalis*, and *C. dubliniensis*. In *C. glabrata*, echinocandin resistance has also been associated with mutations in the *FKS2* gene (Pfaller 2012).

II.5.2. Biofilms

The biofilm state is the preferred mode of growth of microorganisms in natural environments. In the past years, several reports have associated biofilms with over 65% of hospital-acquired infections. It has also been suggested that *Candida* strains with a high ability to form biofilms are generally more virulent than others (Williams, Kuriyama et al. 2011).

The exact mechanism of biofilm resistance to antifungals remains unclear, but it is probably multifactorial. There are three possible mechanisms of biofilm resistance: restricted penetration of drugs through the biofilm matrix; phenotypic changes resulting from a decreased growth rate or nutrient limitation; expression of resistance genes induced by contact with a surface. It has also been suggested that a small number of "persister" cells are responsible for resistance (Douglas 2003).

Regarding the restricted penetration, it has long been supposed that the matrix of extracellular polymeric material might exclude or limit the access of drugs to organisms in the deeper part of the biofilm. To investigate if the matrix plays a role in the resistance of biofilms to antifungal agents, the susceptibility profiles of biofilms where compared between biofilms of *C. albicans* which have relatively little matrix and biofilms of *C. albicans* which produce much more matrix. No significant differences in susceptibility to any of the drugs tested were found, indicating that drug resistance is unrelated to the extent of matrix formation (Baillie and Douglas 2000). However, it had been shown, in another study (Baillie and Douglas 1999), that resuspended cells (which presumably had lost most of their matrix) were some 20% less resistant to amphotericin B than intact biofilms, suggesting that the matrix might play a minor role in drug resistance.

Biofilm cells are known to grow slowly because of the limited availability of nutrients, particularly at the base of the biofilm. A slow growth rate is often accompanied by changes in cell surface composition, which could affect the susceptibility of the microorganisms to antifungal agents. To investigate if growth rate is an important modulator of drug activity in biofilms, the susceptibility of *C. albicans* biofilms to ampB was compared with that of planktonic cells, the both cases with several growth rates. It has been demonstrated that biofilms were resistant to the drug at all growth rates tested whereas planktonic cells were resistant only at low growth rates (Baillie and Douglas 1998). Another study (Baillie and Douglas 1998) demonstrated

that not only the low growth rates, but also other conditions of growth, like glucose and irion availability, can interfere with drug susceptibility. Glucose-limited and iron-limited biofilms, grown at the same low rate, were equally resistant to amphotericin B. Iron-limited biofilms probably resemble the most to biofilms growing *in vivo*, as to the fact there is an abundance of iron in the human body, most of it is located intracellularly or tightly complexed to iron-binding glycoproteins, thus being relatively inaccessible to microorganisms.

Microorganisms that form biofilms express an altered phenotype. To investigate the surface-induced expression of resistance genes, it had been identified genes that are activated or repressed in *Candida* biofilms compared with planktonic cells. Genes coding for multidrug efflux pumps are of particular interest, since the upregulation of these genes results in a multidrug-resistant phenotype. *Candida albicans* possesses two different types of efflux pump, ATP-binding cassette (ABC) transporters and major facilitators, which are encoded by *CDR* and *MDR* genes, respectively (Douglas 2003). A study (Ramage, Bachmann et al. 2002) has demonstrated that genes encoding both types of efflux pump are upregulated during biofilm formation and development. However, mutants carrying single or double deletion mutations in some of these genes were highly susceptible to fluconazole when growing planktonically but still retained the resistant phenotype during biofilm growth. These results strongly suggest that drug resistance in *C. albicans* biofilms is a complex process that cannot be explained by a single molecular mechanism.

However, it has been demonstrated *in vitro* that caspofungin is effective against *C. albicans* and *C. glabrata* biofilms (Cateau, Berjeaud et al. 2001). Caspofungin inhibits the synthesis of 1,3-β-D-glucan, the major structural component of *Candida* cell walls, suggesting that glucan synthesis might be a particularly effective target for biofilms if the biofilm matrix also contains this polysaccharide (Kuhn, George et al. 2002).

II.6. Drug Efflux pumps

There are two main drug efflux pumps classes, the ATP-binding cassette (ABC) transporters and the Major-Facilitator Superfamily (MFS) transporters that are involved

in the resistance to antifungal agents, particularly azoles (Richard D. Cannon, Erwin Lamping et al. 2009).

II.6.1. Major-Facilitator Superfamily (MFS)

The MFS transporters are proteins with transmembrane domains (TMD) subtract specific. These transporters uses an electronic gradient as driving force to efflux the drugs out of the cell (Figure II.10) (Richard D. Cannon, Erwin Lamping et al. 2009).

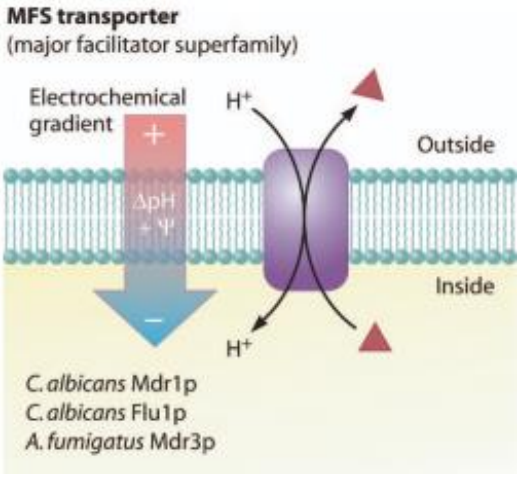

Figure II. 10. Representation of a MFS transporter: efflux of drugs with influx of protons into the cell. Adapted from (Richard D. Cannon, Erwin Lamping et al. 2009).

However, there is a more evident relation with the resistance to azoles and the ABC transporters than with the MFS transporters.

II.6.2. ATP-Binding Cassette (ABC)

The ABC transporters are proteins localized in the cellular membrane and in the organelles membranes that contain TMD substrate-specific and nucleotide-binding domains (NBD). These transporters use the ATP hydrolyses to efflux the drug out of the cell (Figure II.11) (Richard D. Cannon, Erwin Lamping et al. 2009).

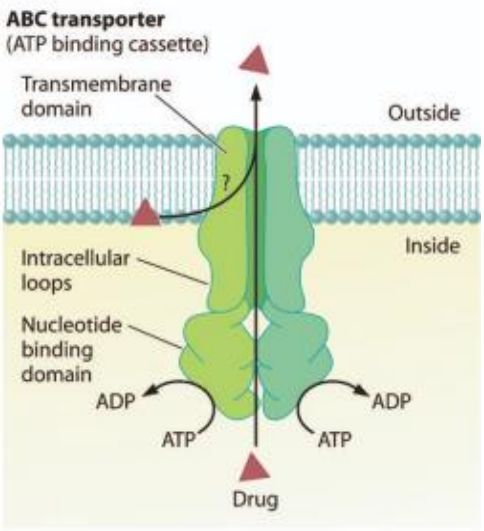

Figure II. 11. Representation of an ABC transporter: efflux of drugs with ATP hydrolyses. Adapted from (Richard D. Cannon, Erwin Lamping et al. 2009).

Regarding the resistance to FLU acquired by *C. glabrata* cells, the transcriptional induction and upregulation of genes encoding ABC transporters (*CDR1*, *CDR2* and *SNQ2*) have been reported (Tscherner, Schwarzmüller et al. 2011; Samaranayake, Cheung et al. 2013). The Figure II.12 illustrates the CDR1 transporter: two identical halves, each with a hydrophilic N-terminal domain which contains units of the ATP-binding (Walker A and Walker B), followed by a C-terminal hydrophobic domain with six transmembrane segments (Richard D. Cannon, Erwin Lamping et al. 2009).

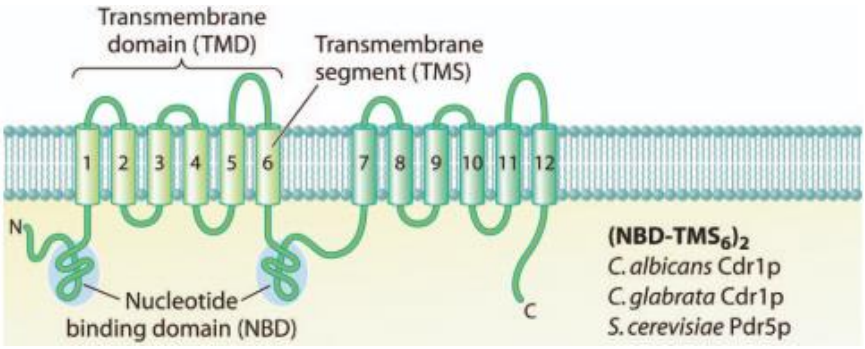

Figure II. 12. Representation of the hydrophilic N-terminal and the hydrophobic C-terminal domains. Adapted from (Richard D. Cannon, Erwin Lamping et al. 2009).

On other hand, a mutation in the gene that encodes a regulator of multidrug transporter genes, *PDR1*, was associated with its upregulation. This fact contributes to upregulation of *CDR1* and *SNQ2* genes (Vermitsky and Edlind 2004). However, little is known about the mechanisms of *C. glabrata* biofilms resistance.

II.7. Ergosterol

Ergosterol is a biomolecule that is one of the main components of fungus cellular membrane. *Candida* grown in presence of azoles has a reduction in the ergosterol content of membranes and also an accumulation of toxic ergosterol precursors, such as 14-α-methylergosta-8,24(28)-dien-3β,6α-diol (Richard D. Cannon, Erwin Lamping et al. 2009).

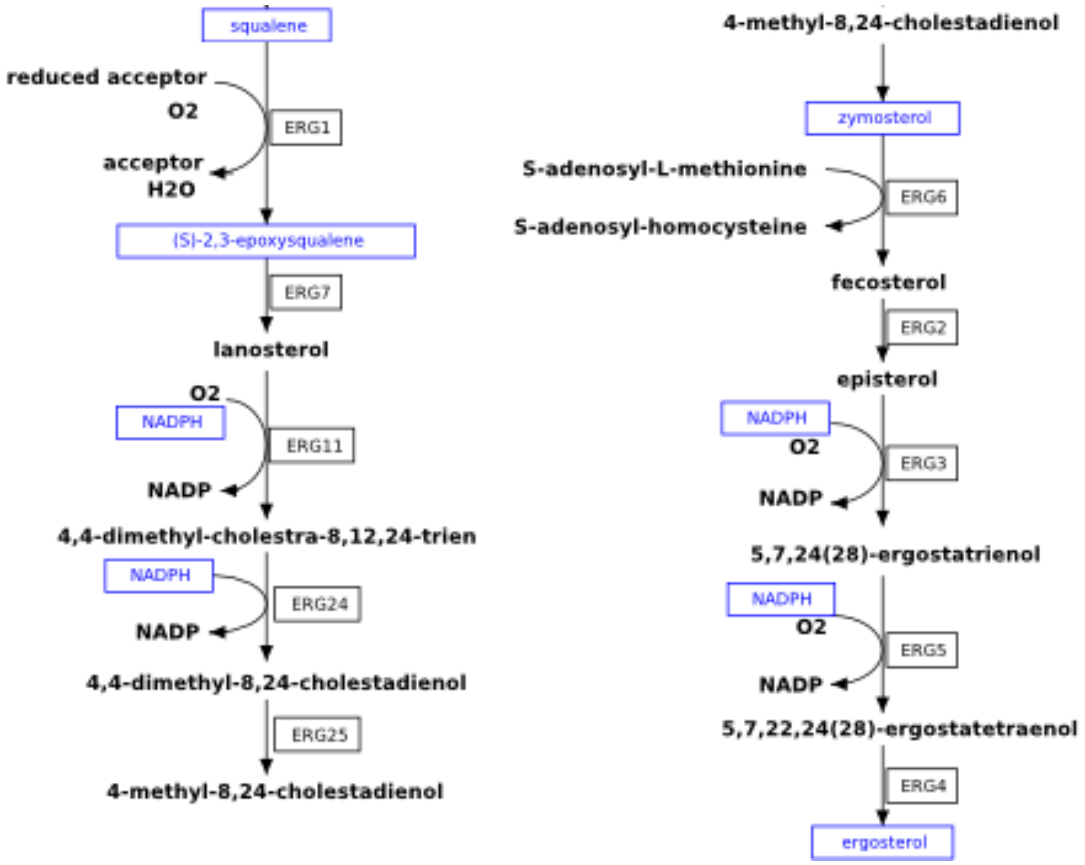

Figure II. 13. Ergosterol biosynthesis pathway. Adapted from Wikipathways 2010 [http://www.wikipathways.org/index.php/Pathway:WP343].

It was been reported an overexpression and mutation of several genes involved in the ergosterol biosynthesis pathway (Figure II.13) as *ERG1*, *ERG3*, *ERG6*, *ERG9* and *ERG11* (Antonia Geber, Hitchcock et al. 1995; Patrick Vandeputte, Guy Tronchin et al. 2007; Richard D. Cannon, Erwin Lamping et al. 2009).

Chapter III

Materials and Methodology

III.1. Organisms and growth conditions

A total of four different *Candida glabrata* strains were used in this work. The reference strain *C. glabrata* 2001 from the American Type Culture Collection (ATCC), two oral isolate (AE2 and D1) from the biofilm group of the Centre of Biological Engineering, originally isolated from Clinic of Dentistry, Congregados, Portugal, two urinary (562123 and 513100) and two vaginal (534784 and 585626) tract isolates, both isolated from patients of the Hospital of S. Marcos, Braga, Portugal. The identity of all isolates was confirmed using CHROMagar Candida (CHROMagar, France) and by PCR-based sequencing using specific primers (ITS1 and ITS4) against the 5.8S subunit gene reference. Genomic DNA was extracted following previously described procedures (Williams, Wilson et al. 1995). The PCRs products were sequenced using the ABI-PRISM Big Dye terminator cycle sequencing kit (Perkin Elmer, Applied Biosystems, Warrington, UK). All *Candida* strains were subcultured on Sabouraud dextrose agar medium (SDA; Merck, Germany) at 37 ^{o}C for 48 h.

III.2. Antifungal susceptibility tests

Minimum inhibitory concentrations (MICs) for fluconazole (FLU; Sigma-Aldrich, USA) were determined using the microdilution method, in accordance with the guidelines of the Clinical Laboratory Standards Institute (CLSI) (M27-A2).

The FLU concentrations tested were of 5, 50, 312.5, 625 and 1250 mg ml^{-1} and were prepared in RPMI 1640 (Sigma-Aldrich, USA). Thus, a small colony of each strain cultured on SDA was suspended in 5 ml of saline solution (NaCl 0.85%) and the cellular density adjusted to turbidity equivalent to a 0.5 McFarland standard in saline buffer. The yeasts suspensions were diluted (1:100) in saline solution and afterward diluted (1:20) in RPMI 1640, according to the standard.

Each *Candida* suspension (100 μl) was added to the respective well of microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium) containing 100 μl of each specific concentration of FLU solutions. Controls without antifungal agents were also performed. The microtiter plates were incubated at 37 ^{o}C, and the MICs values

determined visually as the lowest concentration of FLU showing no yeast growth after 48 h. Additionally, a volume (50 μl) of each cell suspension treated with FLU was recovered to a new well and serial decimal dilutions (in phosphate-buffered saline; PBS 0.1 M pH 7.5: NaCl 0.8%, KCl 0.02%, K_2HPO_4 0.02%, $NaHPO_412H_2O$ 0.285%) were plated onto SDA. Agar plates were incubated for 24 h at 37 ^oC, and the total number of colony forming units (CFUs) was determined. The results were presented per Log_{10} CFU per milliliter (Log_{10} CFU ml^{-1}). The assays were performed in triplicate and on three separate occasions.

III.3. Biofilm formation

An inoculum of each yeast strain, obtained from SDA plates, was suspended in 20 ml of Sabouraud dextrose broth (SDB; Merck, Germany) and incubated at 37 ^oC for 18 h under agitation (120 rpm). Then, the cells were harvested by centrifugation at 3000 g for 10 min at 4 ^oC and washed twice with 15 ml of PBS pH 7.5. Pellets were suspended in SDB and the cellular density adjusted to $2x10^7$ or $1x10^7$ cells ml^{-1} using a Neubauer counting chamber, to use according with each experiment.

III.4. Fluconazole effect against pre-formed *Candida glabrata* biofilms

In order to test biofilms resistance to FLU, *C. glabrata* biofilms were pre-formed during 24 h in SDB. For that, 200 μl of each *Candida* suspension containing $1x10^7$ cells ml^{-1} was added to the respective well of microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium) and incubated at 37 ^oC under agitation (120 rpm). After this time, the medium was totally aspired and the biofilm washed once with 200 μl of PBS to remove non-adherent cells. At this time, the solutions of FLU (at 50, 625 and 1250 mg ml^{-1}) were added to the specific wells and incubated at 37 ^oC for extra 24 h. Controls devoid of FLU were also incubated. The assays were repeated in triplicate on three different occasions.

III.5. Fluconazole effect on *Candida glabrata* biofilm formation

In order to study the effect of FLU in the biofilm formation, FLU was added in the beginning of the formation process. For that 96-wells microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium) were filled with increased concentrations of FLU (at 50, 625 and 1250 mg ml^{-1}) diluted in SDB. At each well containing 100 μl of each specific concentration of FLU was added 100 μl of *Candida* suspension containing $2x10^7$ cells ml^{-1}. The microtiter plates were incubated at 37^o C under agitation (120 rpm). Controls with *Candida* cells and without FLU were also performed. The assays were repeated in triplicate on three different occasions.

III.6. Biofilm analysis

III.6.1. Biofilm biomass determination

Total biofilm biomass was quantified by crystal violet staining methodology (Silva, Henriques et al. 2009). For that, the medium was totally aspirated and the biofilms washed once with 200 μl of PBS to remove non-adherent cells. The biofilms were fixed with 200 μl of methanol and removed after 15 min. The microtiter plates were allowed to dry at room temperature. Then, 200 μl of crystal violet (CV; 1%, v/v) were added to each well. After 5 min, the excess of CV was removed and for that, the biofilms were gently washed twice with water. Lastly, 200 μl of acetic acid (33%, v/v) were added to each well to release and dissolve the CV stain. The absorbance of suspensions was measured at 570 nm and the results were presented as absorbance per unit area (Abs/cm^2). The assays were performed fivefold and on three separate occasions.

III.6.2. Biofilm cultivable cells determination

The number of cultivable cells on biofilms was determined by the enumeration of colony forming units (CFUs). For both cases, the medium was aspired and the biofilms washed once with 200 μl of PBS to remove non-adherent cells. Then, biofilms

were scraped from the wells and the suspensions were vigorously vortexed for 2 min to disaggregate cells from matrix. Serial decimal dilutions in PBS were plated on SDA and incubated for 24 h at 37 ^{o}C. The results were presented as total of CFUs per unit area (Log_{10} CFUs cm^{-2}). The assays were performed in triplicate and on three separate occasions.

III.6.3. Biofilm matrix composition

In order to analyze the matrices composition, polysaccharides and proteins were quantified. For that, 250 µl of *C. glabrata* suspensions ($2x10^7$ cells ml^{-1} in SDB) were placed into 24-wells microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium) containing 250 µl of FLU solutions diluted in SDB to a final concentration of 0, 50 and 1250 mg ml^{-1}. The biofilms were formed during 24 h at 37 ^{o}C under agitation (120 rpm). Then, the medium was totally aspired and the biofilms washed once with PBS to remove non-adherent cells. Biofilms were scraped from the wells, concentrated in PBS and their dry weights were determined by weighting 1 ml of the suspensions. In order to separate the cells from the biofilm matrices, the suspensions were sonicated (Ultrasonic Processor, Cole-Parmer, Illinois, USA) for 30 s at 30 W and centrifuged at 8000 g for 5 min at 4 ^{o}C to recover the supernatants containing the biofilm matrices (Silva, Henriques et al. 2009). Therefore, supernatants were filtered using 0.2 µm filters and used for polysaccharides and proteins quantification. For polysaccharides quantification, 0.5 ml of phenol (50 g l^{-1}) and 2.5 ml of sulfuric acid (95-97%) were added to 0.5 ml of each supernatant, the mixtures were vortexed and the reaction was extended by 15 min at room temperature, according the Dubois method (Dubois, Gilles et al. 1956). The absorbance was read at 490 nm and the total of polysaccharides quantification was determined by extrapolation with a standard curve, using glucose as the standard. For protein quantification, BCA Kit (Bicinchoninic Acid, Sigma-Aldrich, St Louis, USA) was used according to the manufacturer's instructions. The absorbance was read at 562 nm and the proteins quantified by extrapolation by standard curve, using bovine serum albumin (BSA) as the standard. The assays were repeated in triplicate on three different occasions.

III.6.4. Biofilm structure

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

III.7. Gene expression analysis

For the molecular approach only two *Candida* strains were selected, *C. glabrata* ATCC 2001 and *C. glabrata* 562123, the highest and the lowest resistant to FLU, respectively.

III.7.1. Gene selection and primers design for quantitative real-time PCR

Three genes (*CDR1*, *SNQ2* and *PDR1*) were selected to study their expression in planktonic and biofilm cells in the absence and presence of FLU concerning ABC transporters and five genes (*ERG1*, *ERG3*, *ERG6*, *ERG9* and *ERG11*) were selected to study their expression in planktonic and biofilm cells in the absence and presence of FLU concerning the ergosterol biosynthesis pathway. The gene sequences of interest were obtained from *Candida* Genome Database (CGD) and the primers for quantitative real-time PCR (qRT-PCR) were designed using Primer 3 (http://simgene.com/Primer3) web-based software and are listed in Table III. 1. In order to verify the specificity of each primer pair for its corresponding target gene, PCR products were first amplified from *C. glabrata* ATCC 2001 and *C. glabrata* 562123 genomic DNA.

Table III. 1. Primers used for quantitative RT-PCR analysis

Sequence (5'→ 3')	Primer	Target	PCR product size (bp)
TTGTTGGTGTTCCTGGTGAA	Forward	*CDR1*	142
ATGGACCATGCTGTTTGTGA	Reverse		
CGATGCACCAACCAAGTATG	Forward	*SNQ2*	130
ACCACCGACAGTCATCAACA	Reverse		
CGGTGAGTTGGCCCTTACAA	Forward	*PDR1*	171
TTTAATGTCGGCGGTTTCGC	Reverse		
CACCGGTTACACCGTCTTCT	Forward	*ERG1*	121
CAGGACCTTGTCGTTACCGT	Reverse		
CACCCAGTCGACGGTTACTT	Forward	*ERG3*	158
TTGACAACTGGGTTGTTGGA	Reverse		
CTTCGACAAAGTGTACGCGA	Forward	*ERG6*	108
TAAACGGCGAAAGTACCACC	Reverse		
ATTGTCCTTGCAGGTTTTGG	Forward	*ERG9*	119
TCCTCGGCGTAGTCTCTGAT	Reverse		
CTCCATACTTGCCATTCGGT	Forward	*ERG11*	123
CTTCAGTTGGGTAACGCCAT	Reverse		
GTTGACCGAGGCTCCAATGA	Forward	*ACT1*	164
CACCGTCACCAGAGTCCAAA	Reverse		

III.7.2. Biofilm and planktonic cells preparation

Biofilms of the selected strains were grown in 96-wells microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium) in three different conditions – 0 (control), 50 and 1250 mg ml^{-1} of FLU – with a final concentration of $1x10^7$ cells ml^{-1} during 24 h (as described above). After, biofilm formation the medium was aspired and the wells were washed with PBS pH 7.5 to remove non-adherent cells. Biofilms were then scraped from wells with 1 ml of PBS and sonicated (Ultrasonic Processor, Cole-Parmer, Illinois, USA) for 30 s at 30 W to separate the cells from the biofilm matrix. Cells were harvested by centrifugation at 8000 g for 5 min at 4 ^oC. Additionally, planktonic cells were grown in 25 ml Erlenmeyers in the presence of 0, 50 and 1250 mg ml^{-1} of FLU

with a final concentration of $1x10^7$ cells ml^{-1} also during 24 h. Cells were harvested by centrifugation at 8000 g for 5 min at 4 ^oC and were washed once with 10 ml of phosphate buffered saline (PBS; pH 7.5).

III.7.3. RNA extraction

RNA extraction was performed using PureLink® RNA Mini Kit (Invitrogen, Carlsbad, USA).

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

III.7.4. Synthesis of cDNA

To synthesize the complementary DNA (cDNA) the iScript cDNA Synthesis Kit (Bio-Rad, Berkeley, USA) was used according to the manufacturer's instructions. For each sample 10 µl of the extracted RNA was used at a final volume of 50 µl of reaction. cDNA synthesis was performed firstly to 70 ^oC for 5 min and then 42 ^oC for 1h. The reaction was stopped by heating for 5 min at 95 ^oC.

III.7.5. Quantitative Real-Time PCR (qRT-PCR)

Real-time PCR (CF X96 Real-Time PCR System; Bio-Rad, Berkeley, USA) was used to determine the relative levels of *CDR1*, *SNQ2* and *PDR1* mRNA transcripts in the RNA samples, with *ACT1* used as a reference candidal housekeeping gene. Each reaction mixture consisted of a working concentration of SsoFast EvaGreen Supermix (Bio-Rad, Berkeley, USA), 50 µM of forward and reverse primers, and 4 µl cDNA, in a

final reaction volume of 20 µl. Negative controls (water), as well as, non-transcriptase reverse controls (NRT) were included in each run. The relative quantification of genes expression was performed by the Pfaffl method (Pfaffl 2001). Each reaction was performed in triplicate and mean values of relative expression were determined for each gene.

III.8. Statistical Analysis

Results were compared using a two-way ANOVA with the Bonferroni test, using GraphPad Prism 5 software. All tests were performed with a confidence level of 95%.

Chapter IV

Analysis of the results

IV.1. Fluconazole minimum inhibitory concentrations and its effect against *Candida glabrata* biofilms

Minimum inhibitory concentrations (MICs) were determined using the microdilution method and by CFUs counts (Table IV.1). The results showed that all strains were resistant to FLU according to the MIC breakpoint of EUCAST which is 32 mg l^{-1} (EUCAST), and that fungistatic activity was strain dependent (Table IV.1). The lowest values of MIC (40-50 mg l^{-1} and 50-312.5 mg l^{-1}), were obtained for *C. glabrata* 562123 and *C. glabrata* 513100, respectively, both isolated from urinary tract. On the other hand, *C. glabrata* ATCC 2001 exhibited the highest resistance, with MIC values equal or superior to 1250 mg l^{-1}. It was also possible to observe that *C. glabrata* AE2, D1 and 585626 are extremely resistant to FLU, with MIC values ranging from 625 to 1250 mg l^{-1}. *Candida glabrata* 534784 presented an intermediate value of FLU resistance.

Table IV. 1. Minimum Inhibitory Concentrations (MICs) of fluconazole against *Candida glabrata* strains

Strains	MIC (mg l^{-1})
C. glabrata ATCC 2001	≥ 1250
C. glabrata AE2	625 - 1250
C. glabrata D1	625 - 1250
C. glabrata 534784	312.5 - 625
C. glabrata 585626	625 - 1250
C. glabrata 513100	50 – 312.5
C. glabrata 562123	40-50

To determine the susceptibility of biofilms to FLU 24 h biofilms were treated with different concentrations of this agent. Figures IV.1 A and B presented the mean and standard deviation (SD) values of the Log_{10} CFU cm^{-2} and its correspondent total biomass values (Abs_{570} cm^{-2}), for *C. glabrata* biofilms. As expected FLU concentration below the MIC values (50 mg l^{-1}) did not cause any cell reduction and concentrations above the MIC values were also ineffective in controlling the biofilm population

(Figure IV.1 A). The same results were obtained when measuring biofilm total biomass, the exceptions were the strains ATCC 2001 and AE2 that showed a significant biomass reduction ($P<0.01$) in the presence of FLU of 1250 mg l^{-1}. These data demonstrated that biofilms in general are much more resistant to FLU than their planktonic counterpart's cells.

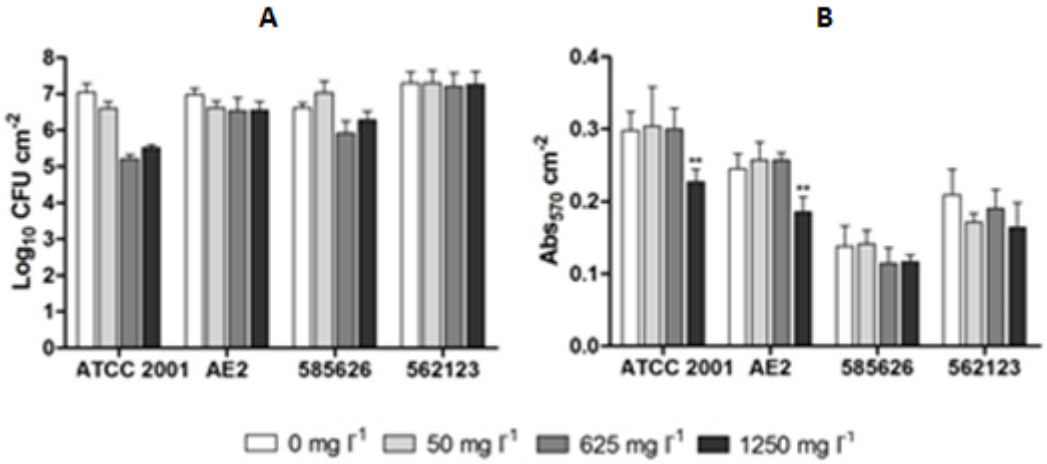

Figure IV.1. Effect of fluconazole on *C. glabrata* pre-formed biofilms. Mean values of the logarithm of colony forming units normalized by unit of area (Log_{10} CFU cm^{-2}) presented on pre-formed biofilms treated for additional 24 h with different FLU concentrations (A); Mean values of the absorbance at 570 nm normalized by unit of area (Abs_{570} cm^{-2}) of *C. glabrata* biofilms treated for additional 24 h with different FLU concentrations (B). Error bars indicate the standard deviations. ** Indicates $P<0.01$ and statistically different from the control.

In order to determine if FLU could prevent biofilm formation, biofilms were formed in the presence different concentrations of FLU (Figure IV.2 A and B). No significant reductions in the number of viable cells were observed for the lower concentration of FLU tested (Figure IV.2 A). Conversely, for higher values of FLU (range 625 mg l^{-1} to 1250 mg l^{-1}), *C. glabrata* ATCC 2001 presented the highest reduction in presence of higher values of FLU applied (range 625 mg l^{-1} to 1250 mg l^{-1}) (Figure IV.2 A). Nevertheless, only a 1.5-log_{10} of reduction was obtained in comparison to the control ($P>0.05$). Figure 2B presents the total biomass values of biofilms of 24 h formed in the presence of different concentrations of FLU. The data showed that higher concentrations of FLU led to lower values of total biomass for all *C. glabrata* strains

tested, with exception of *C. glabrata* 585626 and *C. glabrata* ATCC 2001. Curiously, biofilms of *C. glabrata* ATCC 2001 presented a significant increase in the total biomass values ($P<0.01$) at FLU concentration 50 mg l^{-1} comparatively to the controls (biofilms without FLU). Nevertheless, for the higher concentrations of FLU, 625 and 1250 mg l^{-1}, it was observed a drastic and significant biomass reduction ($P<0.001$). Biofilms of AE2 and 562123 strains presented a significant biomass reduction ($P<0.01$ and $P<0.001$, respectively) at FLU concentrations of 625 and 1250 mg l^{-1}. The total biomass of *C. glabrata* 585626 biofilms was also significantly reduced ($P<0.001$) in presence of FLU concentration of 625 mg l^{-1}.

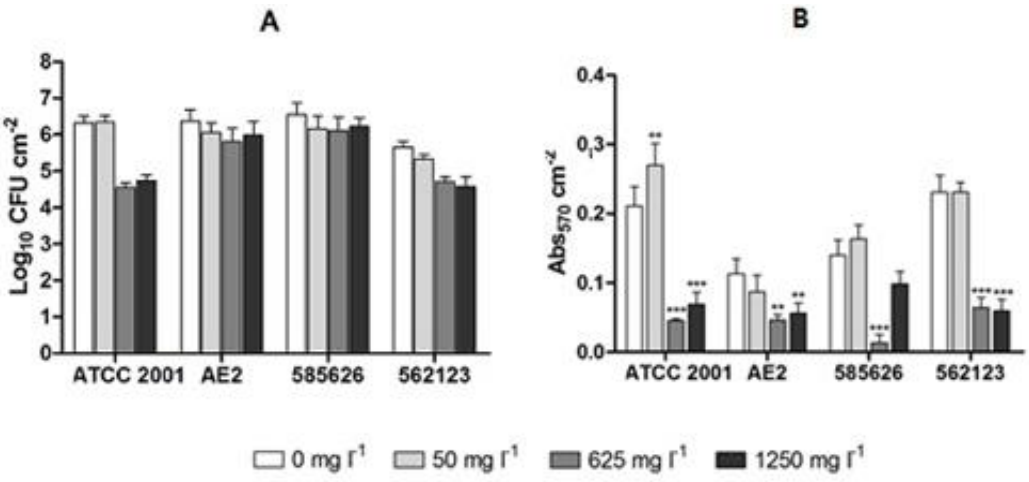

Figure IV.2. Effect of fluconazole on the control of *C. glabrata* biofilms. Mean values of the logarithm of colony forming units normalized by unit of area (Log_{10} CFU cm^{-2}) (A); Mean values of the absorbance at 570 nm normalized by unit of area (Abs_{570} cm^{-2}) (B), on 24 h *C. glabrata* biofilms formed in the presence of different FLU concentrations. Error bars indicate the standard deviations. **and *** indicates $P<0.01$ and $P<0.001$, and consequently statistically different from its controls.

From the data presented it can be concluded that FLU does not impair biofilm cell viability but causes a reduction in biofilm total biomass assessed by CV method. So, it can be hypothesized that FLU might influence the production of biofilm matrix that accounts for the total biofilm biomass. To test that hypothesis, the matrices of the biofilms formed in the presence of FLU were extracted and analyzed. Furthermore the biofilm structures were observed by SEM.

IV.2. Biofilm matrix composition and its structure

For the study of biofilm composition and its structure only the most resistant (*C. glabrata* ATCC 2001) and the least resistant (*C. glabrata* 562123) strains were selected. The polysaccharides composition was determined by the phenol/sulfuric acid method (Figure IV.3A) and the protein content was determined with the BCA Kit (Figure IV.3B). In fact, for both strains, the quantities of polysaccharides increased significantly ($P<0.001$) in presence of the two FLU concentrations (Figure IV.3A). Moreover, the most FLU resistant strain had significantly ($P<0.001$) more polysaccharides than the lowest one. Regarding the presence of proteins in biofilm matrices, a significant increase was only observed for the highest FLU concentration tested, in both strains ($p<0.001$). Interestingly, it was in the lowest FLU resistant strain biofilm matrix that it was found more proteins ($P<0.001$), approximately twice, comparing with the most resistant one, in the presence of 1250 mg l^{-1} of FLU.

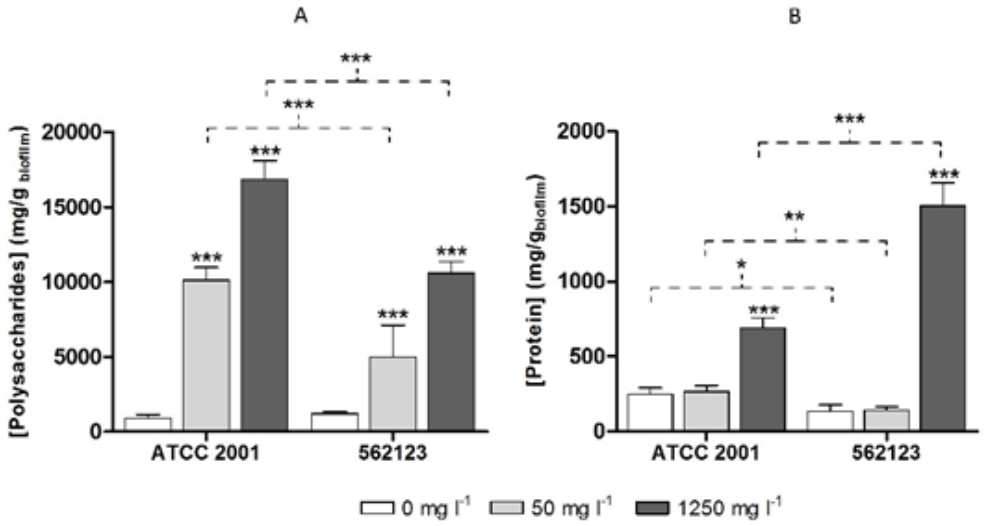

Figure IV. 3. Effect of fluconazole on matrices composition of *C. glabrata* biofilms. Mean values of polysaccharides quantity (A); Mean values of proteins quantity (B), in milligrams per grams of biofilm ($mg/g_{biofilm}$) of dry biofilm grown for 24 h in the presence of different FLU concentrations. Error bars indicate the standard deviations. *, ** and *** indicates $P<0.05$, $P<0.01$ and $P<0.001$, and consequently statistically different from its controls.

SEM analysis was used to examine *C. glabrata* biofilms structure in the presence of FLU, and it is visible that *C. glabrata* biofilms are exclusively formed by

yeasts (Figure IV.4), however with different biofilm structures. The biofilm formed by the reference strain presented a more continuous carpet (Figure IV.4I A), comparatively to *C. glabrata* 562123 (Figure IV.4II A). Interesting, it was observed that, when the biofilms were grown in the presence of FLU, the morphology of biofilms appears like clusters of cells (Figures IV.4I B, IV.4II B).It is also important to refer, that for the maximum FLU concentration, biofilm cells have a more concave aspect and seems to have a reduction on the extracellular matrix (Figure IV.4I C). As it happened with the reference strain biofilm, the formation of cell clusters is seen in the presence of FLU as well as an extracellular matrix reduction (Figures IV.4II B, C). However, the extracellular matrix of the two different biofilms in the presence of FLU seems to be different. This may be explained by the differences in polysaccharides and proteins contents, as showed above.

IV.3. Gene expression of ABC transporters

To study the influence of the biofilm phenotype in the expression of genes encoding for ABC transporters, the same two strains were used (the most and the least resistant).

Figure IV.5 presents the mean n-fold expression levels of *SNQ2*, *CDR1* and *PDR1* in *C. glabrata* ATCC 2001 and in *C. glabrata* 562123 grown as planktonic cells and as biofilm, respectively, in the presence of two concentrations of FLU. In each graph the control bar is the absence of FLU.

The overexpression of genes *SNQ2*, *CDR1* and *PDR1* was observed in planktonic cultures of the strains ATCC 2001 following the application of high concentrations of FLU (Figure IV.5A). This result might explain the high levels of FLU resistance that this strain exhibited (Figure IV.2). Conversely, the levels of expression these genes by *C. glabrata* 562123 planktonic cultures were not affected by FLU. This strain exhibited a 20-fold less resistance to FLU than ATCC 2001 (Table IV.1). Interestingly overexpression of genes *SNQ2*, *CDR1* and *PDR1* was observed in biofilms formed by both *C. glabrata* strains after treatment with FLU, which suggests that FLU might be inducing the expression of ABC transporter genes in the biofilm phenotype of both strains.

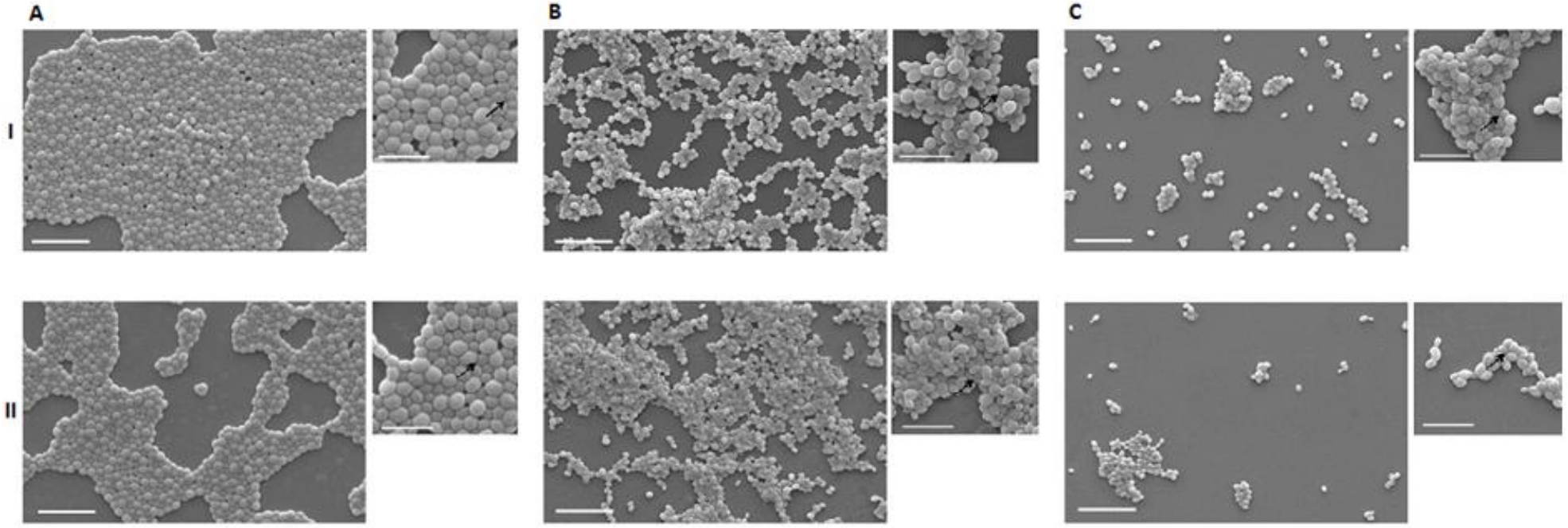

Figure IV. 4. Scanning electron microscopy images of *C. glabrata*. *C. glabrata* ATCC 2001 (I) and *C. glabrata* 562123 (II) biofilms formed in SDB for 24 h in the absence of FLU (A) or in the presence of 50 mg l^{-1} of FLU (B) or 1250 mg l^{-1} (C). The bar in the images corresponds to 20 μm for the magnification 1000x and 10 μm for the magnification of 3000x.

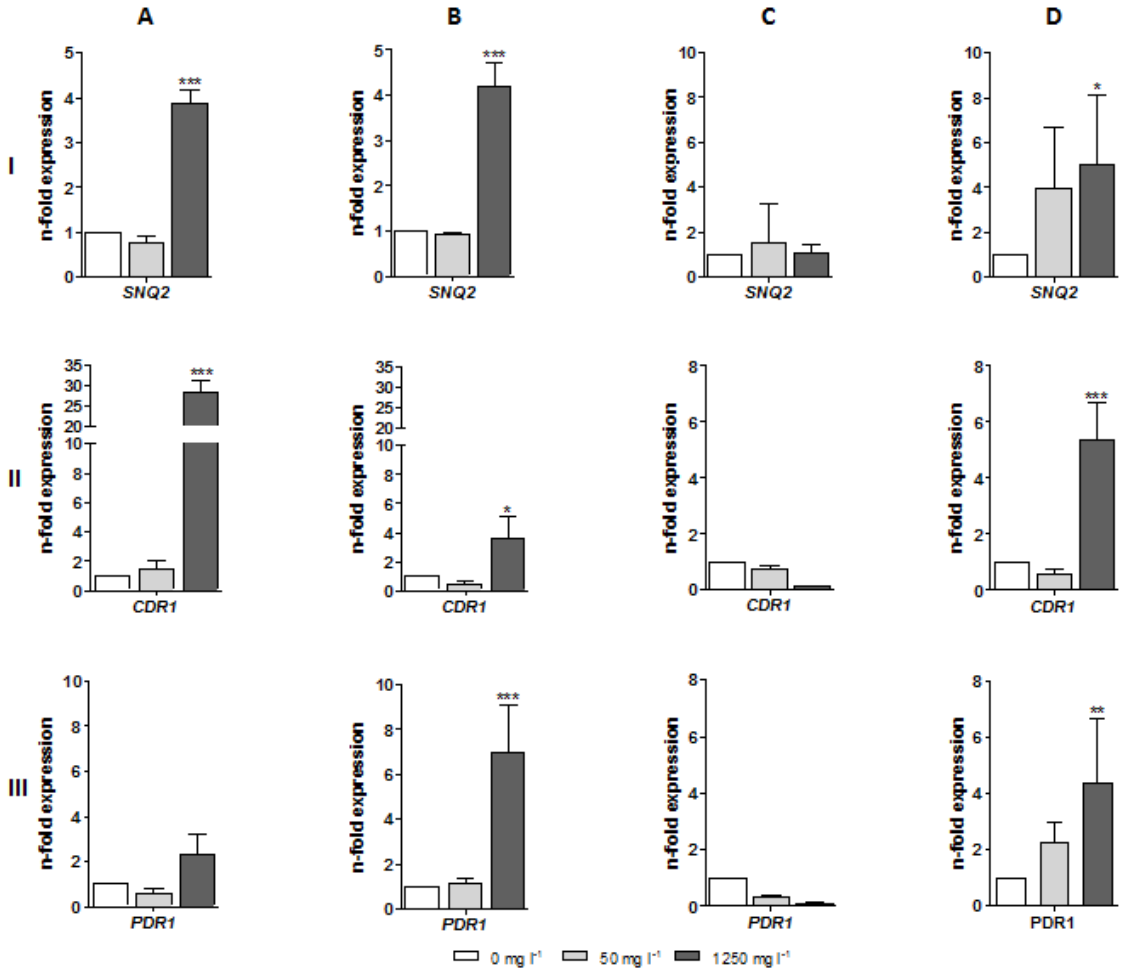

Figure IV. 5. Expression of ABC transporter genes. Mean values of n-fold expression levels of *SNQ2* (I), *CDR1* (II) and *PDR1* (III) genes in *C. glabrata* ATCC 2001 grown as planktonic cells (A) and as biofilm (B) and in *C. glabrata* 562123 grown as planktonic cells (C) and as biofilm (D) treated with 50 and 1250 mg l^{-1} of FLU. Comparisons are made with planktonic and biofilm grown in the absence of FLU. Error bars indicate the standard deviations. *, ** and *** correspond to P<0.05, P<0.01 and P<0.001, respectively.

IV.4. Gene expression of ERG genes

To study the influence of the biofilm phenotype in the expression of genes involved in the ergosterol biosynthesis, the same two strains were used (the most and the least resistant).

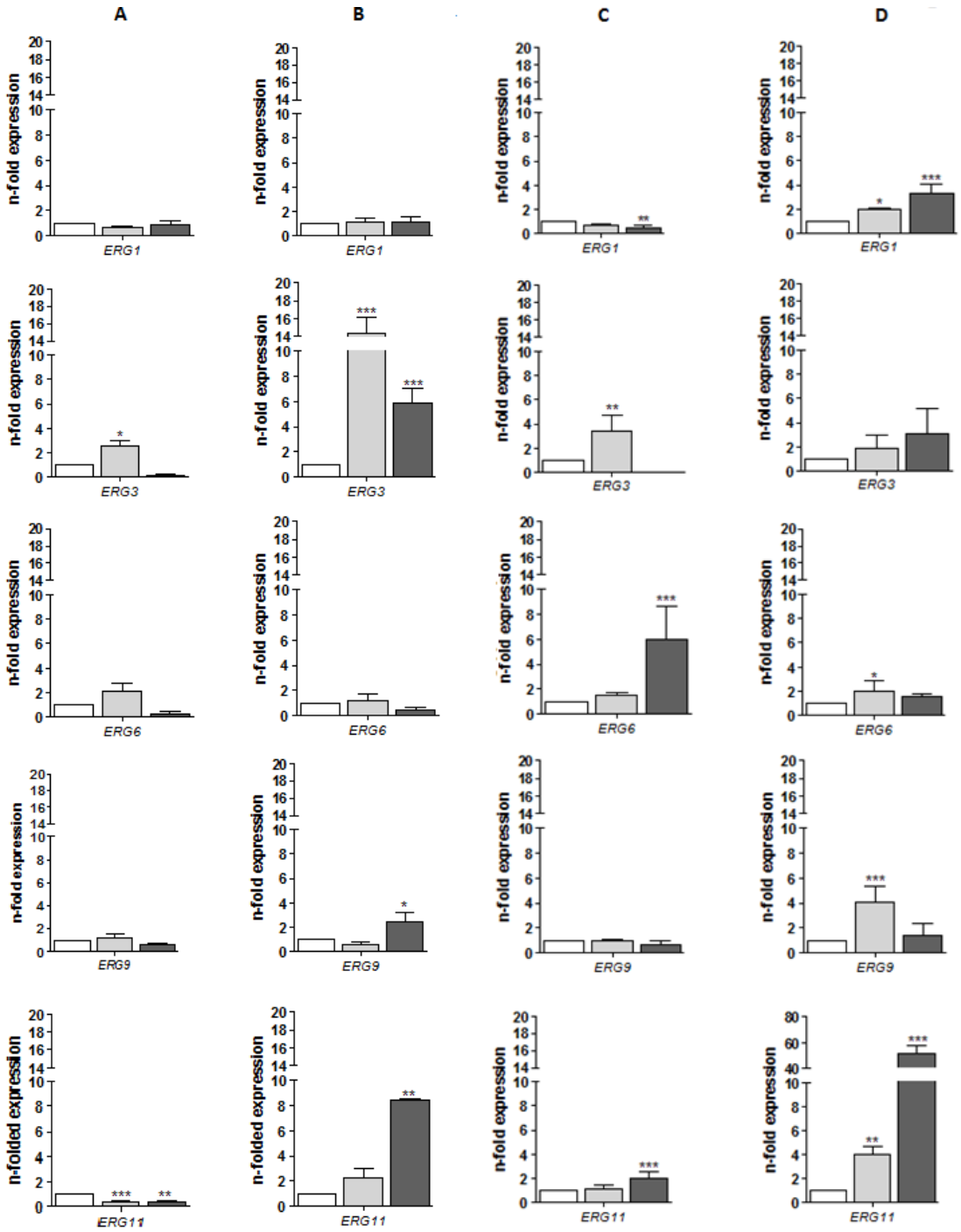

Figure IV. 6. Expression of ERG genes. Mean values of n-fold expression levels of *ERG1*, *ERG3*, *ERG6*, *ERG9* and *ERG11* genes in *C. glabrata* ATCC 2001 grown as planktonic cells (A) and as biofilm (C) and *C. glabrata* 562123 grown as planktonic cells (B) and as biofilm (D) treated with 50 and 1250 mg l^{-1} of FLU. Comparisons are made with planktonic and biofilm grown in the absence of FLU. Error bars indicate the standard deviations. *, ** and *** correspond to $P<0.05$, $P<0.01$ and $P<0.001$, respectively

Figure IV.6 presents the mean n-fold expression levels of *ERG1*, *ERG3*, *ERG6*, *ERG9* and *ERG11* in *C. glabrata* ATCC 2001 and in *C. glabrata* 562123 grown as planktonic cells and as biofilm, respectively, in the presence of two concentrations of FLU. In each graph the control bar is the absence of FLU.

In the most resistant strain (ATCC 2001) no overexpression was observed in planktonic cultures when high concentrations of FLU were added (Figure IV.6A). Interestingly the overexpression of genes *ERG3*, *ERG9* and *ERG11* was observed in planktonic cultures of *C. glabrata* 562123 after treatment with FLU (Figure IV.6B). As the strain 562123 is 20-fold less resistant than the strain ATCC 2001 (Table IV.1), this suggests that the overexpression of *ERG* genes is not the key factor for FLU resistance in planktonic cells.

Additionally, the expression levels of the *ERG3*, *ERG6* and *ERG11* genes in biofilms of *C. glabrata* ATCC 2001 were significantly affected by FLU concentrations (Figure IV.6C). *ERG1*, *ERG9* and *ERG11* genes in biofilms of *C. glabrata* 562123 were also overexpressed (Figure IV.6D).

Chapter V

Discussion of the results

V. 1. Discussion of the results

The present study evaluated the effect of FLU on *C. glabrata* biofilm formation and control, through the quantification of cultivable cells, total biomass and matrix composition, and through the evaluation of the expression of genes involved in FLU resistance.

To reach the goal of this study, antifungal susceptibility tests were performed according to CLSI methodology in order to select the most resistant and sensible strains. For that, the susceptibility of seven strains to FLU was determined and all exhibited resistance to FLU (Table IV.1), however the MIC values were strain dependent. The lowest resistant strain presented a MIC value ranging from 40 to 50 mg l^{-1}. However, a concentration of 1250 mg l^{-1} was required to reduce all visible planktonic cells for three of the seven strains tested. These results are in agreement with other authors, who reported high variability on susceptibility of *C. glabrata* planktonic cells. In a recent study by De Luca (Luca, Guglielminetti et al. 2012) *C. glabrata* strains, presented lower MIC values of 0.25 to 32 mg l^{-1}, while Grandesso (Grandesso, Sapino et al. 2012) reported that the MIC_{90} of *C. glabrata* was a little higher, 64 mg l^{-1}. These differences, which highlight the strain variations, may be due to the strains used, with origin in different clinical isolates recovered from patients submitted to different dose therapies.

It is well known that biofilms are much more resistant to antifungal agents than planktonic cells (Donlan and Costerton 2002), and biofilms are particularly resistant to FLU. Biofilms are the preferred mode of growth of microorganisms in natural environments (Williams, Kuriyama et al. 2011), and are described as surface-associated communities of microorganisms embedded within an extracellular matrix (Silva, Negri et al. 2012). Moreover, it has been reported that clinical isolates are able to form biofilms and have been implicated in high morbidity and mortality rates compared with clinical isolates unable to form biofilms (Silva, Negri et al. 2012). All strains used in this study were able to form biofilms and FLU had no effect on the biofilm cells viability. In fact FLU was not able to reduce cell viability when applied directly on biofilms (Figure IV.1A), and was not able to reduce biofilm formation (Figure IV.2A). These results were in agreement with those reported by other authors (Costerton, Lewandowski et al. 1995; Donlan and Costerton 2002), that showed that the biofilms need about 10 to 100 times more quantity of antifungal agent to be eradicated. Although

FLU did not impair biofilm cell viability it caused a reduction in biofilm total biomass specially when applied in high concentrations, this reduction is probability due to a loss in biofilm matrix (Figure IV.1B and Figure IV.2B). However, despite the reduction of total biomass, the quantities of polysaccharides and proteins undergo a significant increase in the presence of FLU (Figure IV.3). In this way, SEM was use to study the structure and the morphologies of biofilm cells (Figure IV.4). Notwithstanding the inherent destructive nature of the technique, by the possible loss of some cells and matrix during the dehydration process, SEM images demonstrated that the *C. glabrata* biofilms in the presence of FLU, for both strains, results on biofilms structure modifications. Namely, the formation of cell clusters in opposite to biofilms formed in the absence of FLU. This is in accordance with the high levels of polysaccharides and proteins in the extracellular matrix, which was proven to increase cell clustering in biofilms (Mack, Nedelmann et al. 1994; Koo, Xiao et al. 2010). The results point out to the fact that FLU might induce changes in *C. glabrata* biofilm structure and matrix composition. It can be speculated that FLU might be triggering the production and secretion of polysaccharides and proteins that make the biofilm structure more cohesive and less hydrated contributing to a lower uptake of crystal violet, and thus indicating a lower biomass. On the other hand, FLU might be stimulating the expression of genes involved in the efflux pumps, a well-known mechanism of FLU resistance (Vermitsky and Edlind 2004; Tscherner, Schwarzmüller et al. 2011; Samaranayake, Cheung et al. 2013) that contributes to increasing the flow rate inside the biofilm leading to a biofilm structure in cluster form. To test this hypothesis the expression of ABC transporter genes (*SNQ2* and *CDR1*) as well as the zinc finger transcription factor (*PDR1*) that regulates drug efflux pumps was measured in planktonic in biofilm cultures after being treated with FLU.

Indeed, FLU induced the overexpression of the ABC transporter genes of the biofilm cells. It is important to emphasize that these genes were not overexpressed in the planktonic cultures of the less resistant strain. These transporter genes are associated with the mechanism of FLU resistance in *C. glabrata*. Indeed, studies of Burn (Brun, Bergès et al. 2004) and Vermitsky & Edlind (Vermitsky and Edlind 2004) had demonstrated an overexpression of both *CDR1* and *CDR2* (*PDH1*) in *C. glabrata* FLU resistant mutants, although the FLU resistance in *C. glabrata* planktonic cells was mainly due to *CDR1* upregulation (Brun, Bergès et al. 2004). Sanguinetti (Sanguinetti, Posteraro et al. 2005) had also demonstrated the upregulation of *CDR1* and *CDR2* and,

a slight upregulation of *SNQ2* in *C. glabrata* clinical isolates in the presence of FLU. Moreover, Sanglard (Sanglard, Ischer et al. 2001) showed that expression of *SNQ2* was little affected by azole resistance in clinical mutant strains of *C. glabrata*. In our study, an upregulation of *SNQ2* was detected but only in *C. glabrata* ATCC 2001 planktonic cells. Nevertheless, *SNQ2* was express seven times less than *CDR1*, which is in accordance with Sanglard et al. 2001 evidences. Conversely to what has been described in literature, one of to the strains (*C. glabrata* 562123, the least resistant strain) was not able to increase *CDR1* expression in the presence of high concentration of FLU in the planktonic state. Moreover, the expression of *SNQ2* and *PDR1* was downregulated, demonstrating incapacity of the planktonic clusters to respond to FLU by increasing the efflux pumps. Nevertheless, and as said above, these genes were overexpressed in the biofilm phenotype, rendering the biofilm much more resistant to FLU. This fact can be due to the diffusional limitation of FLU inside the biofilm matrix decreasing the amount of FLU that enters in contact with the biofilm cells. This way cells receive less concentration of FLU and have time to develop the mechanisms of resistance.

Regarding the expression of *ERG* genes, the *ERG11* gene plays an unquestionable role in FLU resistance of *C. glabrata* biofilms (Figure IV.6) (Antonia Geber, Hitchcock et al. 1995; Patrick Vandeputte, Guy Tronchin et al. 2007; Richard D. Cannon, Erwin Lamping et al. 2009) and, depending on the strain and in the FLU concentration, some of the other *ERG* genes may be overexpressed to increase resistance of the biofilm.

Chapter VI

Conclusions

VI.1. Conclusions

This study showed that the pattern of resistance to FLU is strain dependent. Moreover it was also demonstrated that biofilms of *C. glabrata* are much more resistant to FLU than planktonic cultures. One of the possible mechanisms of resistance is the overexpression of efflux pumps. The upregulation of the genes encoding for the efflux pumps influences the biofilm structure maybe by creating water channels through the biofilm structures creating cell clusters. This work showed for the first time that ABC transporter genes are implicated in *C. glabrata* biofilm tolerance to high FLU concentrations and influences biofilm structure and that the overexpression of some *ERG* genes is crucial to the resistance of these biofilms.

Chapter VII

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VII.1. References

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