Universidade de Aveiro
Ano 2018

Departamento de Ciências Médicas

Catarina Alexandra Gomes Brinco

Evolution of antifungal drug tolerance in *Candida albicans*

Evolução de tolerância a antifúngicos em *Candida albicans*

Declaração

Declaro que este relatório é integralmente da minha autoria, estando devidamente referenciadas as fontes e obras consultadas, bem como identificadas de modo claro as citações dessas obras. Não contém, por isso, qualquer tipo de plágio quer de textos publicados, qualquer que seja o meio dessa publicação, incluindo meios eletrónicos, quer de trabalhos académicos.

Universidade de Aveiro
Ano 2018

Departamento de Ciências Médicas

Catarina Alexandra Gomes Brinco

Evolution of antifungal drug tolerance in *Candida albicans*

Evolução de tolerância a antifúngicos em *Candida albicans*

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Doutora Ana Rita Macedo Bezerra, investigadora do Departamento de Ciências Médicas da Universidade de Aveiro

This work was supported by the Portuguese Foundation for Science and Technology, POCI- COMPETE2020 and FEDER through grants PTDC/IMI-MIC/5350/2014 and UID/BIM/04501/2013.

o júri

presidente

Doutor Bruno Bernardes de Jesus
professor auxiliar do Departamento de Ciências Médicas da Universidade de Aveiro

Doutor João Manuel Salvador Simões
Investigador da Unidade Genómica do Biocant

Doutora Ana Rita Macedo Bezerra
Investigadora do Departamento de Ciências Médicas da Universidade de Aveiro

agradecimentos

Ao longo do desenvolvimento deste trabalho foram muitas as pessoas que me acompanharam e às quais devo o meu sincero agradecimento por todo o apoio e confiança.

Em primeiro lugar, agradeço ao professor Dr. Manuel Santos por ter permitido que este trabalho fosse possível. À Dra. Ana Rita Bezerra pela orientação ao longo do último ano, por ter sempre demonstrado disponibilidade, paciência e por tudo aquilo que me ensinou.

Agradeço à Dra. Catarina Almeida por se ter disponibilizado para ajudar na citometria de fluxo.

Agradeço ainda aos meus colegas do laboratório pela simpatia e por se terem sido sempre prestáveis em ajudar em qualquer dúvida.

Um dos maiores agradecimentos vai para a minha família, nomeadamente a minha mãe, pelo apoio em todas as horas e por ter sempre acreditado em mim. Agradeço igualmente ao meu pai, ao meu irmão e restante família pelas palavras amigas e de incentivo no momento certo.

Não podia deixar de agradecer a todos os meus amigos e ao meu namorado pelo apoio, preocupação e carinho e por não me deixarem desmotivar nunca.

Muito obrigada!

palavras-chave

Candida albicans, fluconazol, erros de tradução, tolerância a drogas, resistência a drogas, concentração inibitória mínima (MIC).

resumo

Nos dias de hoje, as infeções fúngicas representam um grave problema de saúde pública devido ao aumento da resistência a tratamentos e à pouca variedade de antifúngicos disponíveis.

Candida albicans é o fungo patogénico que mais causa infeções superficiais bem como graves infeções sistémicas que estão associadas a elevadas taxas de mortalidade. Este fungo tem uma característica particular, pois possui um tRNA ($tRNA_{CAG}^{Ser}$) híbrido responsável pela ambiguidade do codão CUG, que pode ser decodificado tanto como serina ou leucina, com níveis de incorporação de 97% e 3%, respetivamente. Já foi demonstrado que na presença de antifúngicos, este nível de incorporação de leucina pode subir até 20% e que as estirpes com maior erro de tradução têm uma maior tolerância à ação de drogas, nomeadamente aos azóis, na qual se inclui o fluconazol. Neste estudo, testamos a hipótese de que os erros de tradução podem estar diretamente associados com o aparecimento de sub-populações de células de *C. albicans* que são tolerantes à droga e a partir das quais, ao longo de tratamentos prolongados, a resistência pode emergir.

De modo a perceber de que forma os antifúngicos podem ou não selecionar sub-populações de células tolerantes, foram feitas competições *in vitro* entre estirpes controlo e estirpes com elevado erro de tradução. Estas competições foram evoluídas experimentalmente ao longo de 400 gerações na presença e ausência de fluconazol. De modo a distinguir as populações, a estirpe controlo (T0) foi marcada com mCherry enquanto que as estirpes com erros de tradução foram marcadas com GFP. Os resultados obtidos mostraram que ao longo da evolução, na presença de antifúngico, houve um aumento do número de células com erro de tradução, sem que isso se resultasse num aumento da concentração inibitória mínima (MIC). Por outro lado, surgiram microcolónias (constituídas exclusivamente por células com elevado erro de tradução) dentro da elipse de inibição do E-test, o que sugere o aparecimento de um fenótipo de tolerância em vez de resistência.

Estes resultados sugerem que as terapias prolongadas com antifúngicos podem selecionar clones com elevado erro de tradução que sustentam o aparecimento e persistência de células tolerantes dentro da população. Este fenótipo de tolerância pode não ser detetado através de alterações significativas da MIC, no entanto, é um factor a ter em consideração, pois pode conduzir ao desenvolvimento de candidíases recorrentes.

keywords

Candida albicans, fluconazole, mistranslation, drug tolerance, drug tolerance, drug resistance, minimum inhibitory concentration (MIC).

abstract

Nowadays, fungal infections are a serious public health issue due to the increasing drug resistance and small number of antifungals available.

Candida albicans is the most prevalent human fungal pathogen, causing invasive fungal infections that are associated with high mortality rates. This fungus has a particular hybrid tRNA ($tRNA_{CAG}^{Ser}$) that is recognized by both the leucyl - and the seryl-tRNA synthetases (LeuRS and SerRS), allowing for incorporation of leucine (3%) and serine (97%) at CUG positions.

It has already been shown that in the presence of antifungals, the level of Leu misincorporation increases up to 20% and that hypermistranslating strains have higher tolerance to drugs, namely azoles, which includes fluconazole. In this study, we tested the hypothesis that mistranslation could be directly linked with the appearance of drug tolerant subpopulations of *C. albicans* cells from which, with prolonged drug treatment, resistance may emerge.

In order to understand if antifungals can select subpopulations of tolerant cells, we carried out *in vitro* competition experiments with fluorescently tagged *C. albicans* strains that were experimentally evolved with or without fluconazole during 400 generations. A wild-type strain (T0) was tagged with mCherry while hypermistranslating strains were tagged with GFP which allowed strain differentiation within the competition.

Results showed an increase of hypermistranslating cells during evolution in the presence of the antifungal, but no significative alteration of the minimal inhibitory concentration (MIC) value was detected. On the other hand, microcolonies (constituted exclusively by hypermistranslator cells) appeared within the inhibition ellipse of the E test, suggesting the emergence of tolerance instead of resistance. These results suggest that prolonged antifungal therapy may select hypermistranslating clones that drive the appearance and persistence of tolerant cells within the population. Tolerance phenotypes may not be detected through significative MIC alterations but it must be taken into consideration since it could be associated with recurrent candidiasis.

LIST OF CONTENTS

List of figures

List of tables

List of abbreviations

- aaRS – aminoacyl-tRNA synthetase
- *C. albicans* – *Candida albicans*
- CO_2 – carbon dioxide
- DNA – deoxyribonucleic acid
- FLC - fluconazole
- g (mg, µg, ng) – gram (milligram, microgram, nanogram)
- GFP – Green fluorescente protein
- GTP – Guanosine 5'-triphosphate
- HIV – Human Immunodeficiency virus
- L (mL, µL) – liter (mililiter, microliter)
- LOH – loss of heterozygosity
- MIC -minimum inhibitory concentration
- mRNA – messenger ribonucleic acid
- MTL - mating type like
- OD - optical density
- PBS - phosphate-buffered saline
- RNA – ribonucleic acid
- Rpm -revolutions per minute
- tRNA – transfer ribonucleic acid
- yEGFP – yeast-enhanced green fluorescent protein

1 Introduction

1.1 Protein Synthesis

By the year of 1953 the DNA three-dimensional structure was already known (proposed by Watson and Crick) as well as the DNA as the substance material of genes. However, the conversion from genes to proteins and how the information was transmitted was not clear yet [1].

The expression of genes is now considered to be a two-stage process, beginning with transcription and the biogenesis of a mRNA, and followed by translation of that mRNA into protein. In the process called translation, information encoded as nucleic acids in mRNA migrates from the nucleus to the ribosomes, where is converted in amino acids and subsequently, proteins [2]–[4].

The process of mRNA translation happens on the ribosome in three phases: initiation, elongation and termination. The initiation phase requires mRNA, initiator methionyl tRNA for the recognition of the initiation codon (AUG) and the small and large ribosome subunits to get together into an initiator complex [5]. The initiation phase also depends on the presence of initiation factors (eIF) that play several roles including the detection of the correct initiation codon [6].

Elongation is the phase of translation in which ribosomal activity generates a polypeptide chain based on the codon sequence of the mRNA. Ribosomes contain three tRNA bindings sites: the acceptor site (A site), the peptidyl site (P site) and the exit site (E site) [3].

With the exception of initiator aminoacyl-tRNAs, that bind the P site, all other aminoacyl-tRNAs (section 1.2) bind the A site. A cognate aminoacyl-tRNA linked to elongation factor Tu (EFTu) binds to its cognate codon of the mRNA in the ribosome at the A site, next to the tRNA in P site, which is bound to the nascent polypeptide. Then, GTP is hydrolysed by EFTu, and the aminoacyl-tRNA is released into the A site if there is correct codon–anticodon pairing. Subsequently, the nascent polypeptide in the P site forms a peptide bond with the amino acid linked to the tRNA in the A site resulting in a peptidyl-tRNA in the A site that is one amino acid longer. The ribosome then translocates one codon along the mRNA, moving the tRNA into the P site to the e site, the peptidyl-tRNA from the A site to the P site and leaving the A site free to accept the next incoming aminoacyl-tRNA (Figure 1) [3].

The termination phase of polypeptide synthesis occurs when a stop codon appears at the ribosome A site causing the disjunction of the translation machinery complex and the release of the finished polypeptide. For this to happen, there has to be a release factor (eRF1) that promotes the hydrolysis of peptidyl-tRNA in response to any of the three stop codons, UAA, UAG or UGA [6], [7].

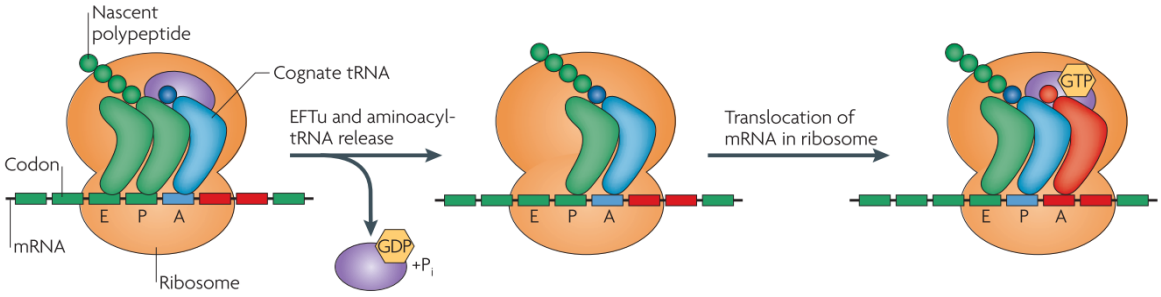

Figure 1. Formation of a polypeptide in the ribosome. Adapted from *N. M. Reynolds, B. A. Lazazzera, and M. Ibba, "Cellular mechanisms that control mistranslation,"* Nat. Rev. Microbiol., *2010.*

1.2 The genetic code: mRNA translation rules

The genetic code was proposed by Crick in 1966, as a universal, non-overlapping triplet code. It is defined by rules, through which the nucleotides are translated into amino acids. There are four distinct ribonucleotide bases designated adenine (A), guanine (G), uracil (U) and cytosine (C) organized in nucleotide triplets, generating 64 possible codon combinations. 61 of them are translated into the 20 standard amino acids and 3 are stop codons [8], [9]. In addition to those, there are two well-characterized noncanonical amino acids: selenocysteine (Sec: 21st amino acid), which is present in varying sets of proteins in diverse organisms from all three domains of life, and pyrrolysine (Pyl: 22nd amino acid) , currently reported only in some archaea [10], [11].

The basic structure of the code and the majority of codon assignments are shared among all life forms. Nonetheless, there are several reports of variations in the genetic code continuing to emerge. The majority of these codon reassignments involve sense to nonsense codon changes (or vice versa) and occur in mitochondria. Only one nuclear sense-to-sense alteration is known so far, namely the reassignment of the CUG codon from leucine to serine in several fungal species of the CTG clade [12].The most common modification described is the reassignment of the UGA stop codon to Trp (UGG codon) which has been identified in many mitochondria as well as some bacteria and ciliates. This

occurs in consequence to a mutation of the CCU anticodon of the tRNA-Trp that creates a UCU anticodon that can pair with both UGA and UGG [10], [13].

Most genetic code alterations are mediated by structural changes in the protein synthesis machinery, particularly tRNAs, aminoacyl-tRNA synthetases (aaRSs), elongation and termination factors. In fact, tRNA and aminoacyl-tRNA synthetases are two important molecules involved in the translation that assure the accurate decoding of an mRNA sequence, by other words, the incorporation of the cognate amino acid in response to a given codon [10]. tRNAs have a dual role in the process of translation as they base pair with the codons of the mRNAs and are aminoacylated by aminoacyl-tRNA synthetases. This amino acid charging activity defines in fact the genetic code as it links anticodons to amino acids [4].

The reaction through which a tRNA is charged with the cognate amino acid is called aminoacylation and directly depends on the activity of aaRSs to catalyse the reaction. Each aaRS recognizes its tRNAs according to some of their nucleotides called identity elements (IEs). Aminoacylation occurs in two steps, namely amino acid activation and tRNA charging [14], [15]. First, ATP and the amino acid bind to the active site of the aaRS to form an aminoacyl-adenylate and then the amino acid is attached to the tRNA by 3'-esterification [16]. Fidelity of translation is highly dependent on the accuracy of tRNA aminoacylation.

1.3 Mistranslation

Errors can happen during any process involved in the conversion from gene to protein. During DNA replication, nucleotide misincorporations occur about one per 10^8 nucleotides and during mRNA transcription the error increases to one per 10^5. Errors in those process can lead to changes on the sequence of codons and compromise the formation of the correct amino acid [17].

However, the process where errors are more common is the translation itself. During translation, several sequential molecular recognition events have to happen, each of which has an inherent error rate. The cells must assure the translational accuracy at the same time that guarantees the translational speed and for that, cells tolerate levels of mistranslation of approximately one error per 10^3 to 10^5 amino acids in order to not compromise the speed of the process. The type of errors that can occur are: tRNA charging with a noncognate amino acid by an aaRS or ribosomal decoding errors [17].

The synthesis of aminoacyl-tRNA is the first step where errors can happen [3]. Mischarging of tRNAs may be caused by the failure of aaRSs to recognize their cognate tRNAs or by the activation of incorrectly bound amino acids. To minimize mischarging, some aaRSs have editing mechanisms that discard chemically similar amino acids from their active sites [18], [19].

Codon decoding errors are mainly caused by misreading of sense codons (missense errors) and nonsense codons (nonsense errors) by near cognate or noncognate tRNAs. The result is the synthesis of mutant proteins. Other causes for synthesis of mutant proteins are the loss of the reading frame (frameshifting), that alters the reading frame to the -1 or to the +1 frames by tRNA slippage during elongation, or by the premature ribosome drop off from the mRNA (processivity errors). Together, these errors result in premature translation termination and synthesis of truncated polypeptide [20].

For a long time, mistranslation has been considered an abnormal cellular process, however there are recent evidences suggesting that cells not only tolerate certain levels of mistranslation, but induce it themselves under some circumstances with important and even desirable biological consequences [17], [21].

Per example, *Mycoplasma* parasites have high inherent levels of mistranslation, namely point mutations and deletions that occur in the editing domains of their ThrRS, LeuRS and PheRS reducing the fidelity of tRNA aminoacylation and resulting in high levels of mistranslation. This constitutive mistranslation helps the *Mycoplasma* create cell surface variability and escape from the host immune system, which constitutes an advantage and gives this parasite similar features to those described in pathogenic organisms [17].

On the other hand, mutations in the editing domain of alanyl-tRNA synthetase (AlaRS) leads to global misincorporation of serine at alanine codons and causes severe neurodegeneration and ataxia in mice [22]. In *E. coli*, similar mutations in the editing mechanism of valyl-tRNA synthetase (ValRS) and isoleucyl-tRNA synthetase (IleRS) decreases bacterial growth rate and viability [23], [24].

1.3.1 Mistranslation in *Candida albicans*

Some species of the *Candida, Debaryomyces and Lodderomyces* genera (CTG clade) show one particular genetic code alteration, in the decoding of the standard leucine (Leu) CUG codon as serine (Ser), mediated by a serine t-RNA that acquired a leucine 5'-CAG-3' anticodon (ser-tRNA$_{CAG}$).The *C. albicans* tRNA is represented on Figure 2 and is an hybrid molecule that has the body of a serine tRNA and the anticodon arm of leucine tRNAs. This molecule contains identity elements for SerRS, namely the three G–C base pairs represented in red on the figure and the discriminator base (G_{73}), and for the LeuRS, A_{35} and m^1G_{37} in the anticodon loop. Therefore, the ser-tRNA$_{CAG}$ can be charged with both leucine (~3%) and serine (~97%), which means that either leucine or serine could be inserted during ribosome decoding [25]–[28].

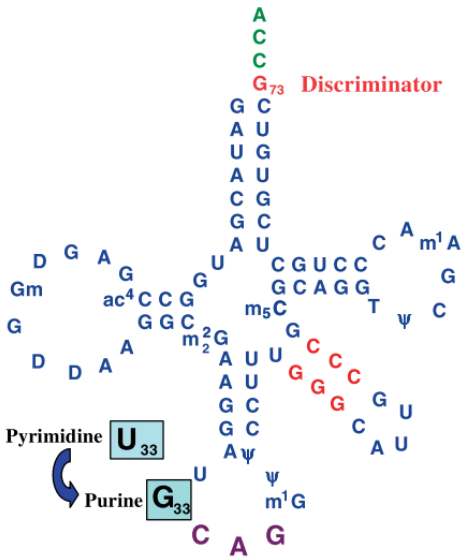

Figure 2. Secondary structure of *C. albicans* Ser-tRNA$_{CAG;}$ Replacement of G_{33} with U_{33} increases leucylation efficiency of the Ser-tRNA$_{CAG}$ *in vitro*, showing that the main function of G_{33} is to keep CUG ambiguity at low level. Adapted from *I. Miranda et al. "Evolution of the genetic code in yeasts,"* Yeast*, vol. 23, no. 3 (2006).*

Previous works from the host laboratory have shown that Ser and Leu incorporation levels at CUG sites in *C. albicans* is very variable. Gomes and colleagues measured leucine misincorporation at CUG codons and showed that *C. albicans* white cells grown at 30ºC have 2,96% of mistranslation. When growing at 37ºC, with hydrogen peroxide or pH 4.0, cells had ambiguity levels of 3,9%, 4,03% and 4,95%, respectively. It has also been seen that even small differences in leucine misincorporation have large effects on *C.albicans* proteome expansion and diversity contributing to generate important phenotypic diversity [11].

Another work engineered *C. albicans* strains to misincorporate increasing levels of Leu at CUG codons (from 0,6% to 98%). Tolerance to the misincorporations was very high, and one strain was able to complete revert the CUG identity from Ser back to Leu (T2KO2). The consequences of the increasing levels of Leu misincorporation were the decreased growth rate compared with the wild type cells, that misincorporate leucine at 3% to 5% under normal stress conditions. On the other hand, increased mistranslation has conferred phenotypic diversity, an impressive tolerance to drugs (particularly fluconazole) and has modulated the host immune cell responses. All these alterations were accompanied with genomic alterations, including mutations and loss of heterozygosity (LOH) relative to the control strain of *C. albicans*. There was a near-complete LOH in a 300-kb region on chromosome V and the entire chromosome R in all strains with a level of leucine misincorporation higher than 50% [27].

Miranda *et al.* have shown that the increased rate of leucine incorporation at CUG decoding triggers *C. albicans* virulence attributes, such as morphogenesis, phenotypic switching and adhesion, suggesting that the protein diversity caused by the ambiguity/variable translation of CUG codon has important functional consequences for the interactions of *C. albicans* with the host. Namely, CUG mistranslation increases the adherence to the host substrates and masks the fungal cell wall molecule β-glucan that is normally recognized by the host immune system, delaying its response [29].

1.4 *Candida albicans* biology

Candida albicans is present on the human microflora as a diploid yeast of mucosal surfaces and is commonly found in the human gastrointestinal, respiratory and genitourinary tracts of approximately 70% of healthy individuals. It generally lives in a commensal way with the host and causes no harm. However, under specific conditions it can turn into an opportunistic pathogen if the host defences are compromised [30]–[33].

One of the hallmark features of *C. albicans* is its phenotypic plasticity that promotes adaptation inside the host. This plasticity includes growing with distinct morphologies, yeast, hyphae, pseudohyphae and chlamydospores (Figure 3). Yeast cells have a round/oval shape, similar to *Saccharomyces cerevisiae* morphology that contrasts with the thin and tube-shaped morphology of hyphal cells. Ellipsoid-shaped pseudohyphal cells share features of both yeasts and hyphae and chlamydospores are large, spherical, thick- walled cells observed *in vitro* under stress conditions, such as starvation and hypoxia [34].

The switch that occurs between the budding yeast and the filamentous forms (pseudohyphae or hyphae) is called dimorphism, being one of the most relevant virulence attributes in *C. albicans.* Both morphological forms seem to have distinct functions during the different stages of infection, including adhesion, invasion, damage, dissemination, immune evasion and host response. The hyphal form has been shown to be more invasive while the smaller yeast form is believed to be mainly involved in dissemination [33], [35]–[37].

C. albicans morphology is also tightly regulated by a network of signal transduction pathways in response to environmental stimuli, per example, at low pH (<6) *C. albicans* cells predominantly grow in the yeast form, whereas at a high pH (>7) hyphal growth is induced. Other conditions that induce hyphal growth include starvation, the presence of serum or N-acetylglucosamine, physiological temperature and CO_2 [35], [37], [38].

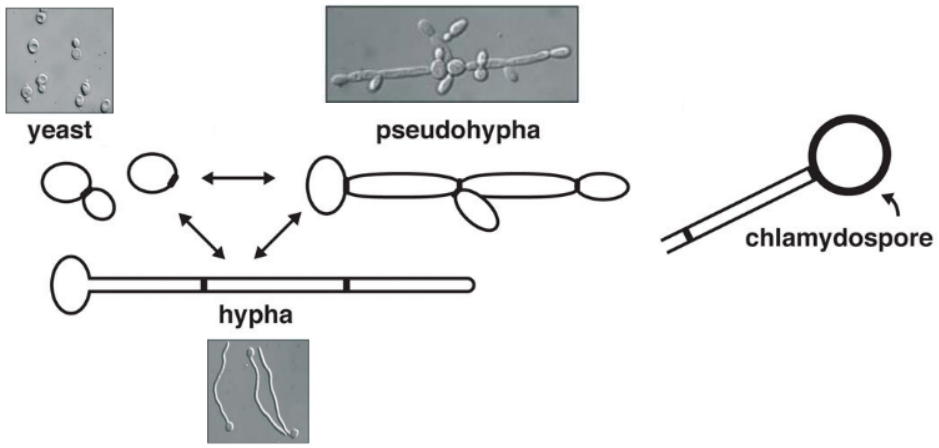

Figure 3. *C. albicans* reversible transitions between yeast, hypha, and pseudohypha cell types. Chlamydospores are generated by terminal (suspensor) cells of mycelia (multicellular hyphae or pseudohyphae) under adverse growth conditions. Adapted from S. M. Noble *et al*., "*Candida albicans cell type switches and functional plasticity in the mammalian host*", 2017 and *J. Kim and P. Sudbery, "Candida albicans, a major human fungal pathogen,"* J. Microbiol*., 2011.*

White-opaque switching is another well described morphological transition, in which white cells are round and generate smooth hemispherical colonies whereas opaque cells are elongated and form flat and grey colonies [33].

For a long time, *C. albicans* was classified as asexual because there was no evidence of the occurrence of mating or meiosis [39]. However, it is already been shown that *C. albicans* has an unusual parasexual mating cycle that shows several adaptations and some similarities to that found in model yeasts like *Saccharomyces cerevisiae* [40].

One of the particularities of mating in *C. albicans* is that is not only regulated by transcriptional factors encoded by the mating locus, but also by an epigenetic switch between “white” and “opaque” phenotypic states [40], [41].

Natural isolates of *C. albicans* are diploid (2N), and those diploid cells are usually heterozygous at the mating type locus (MTL) in an a/α state. These cells have a heterodimer that represses the mating functions and the white-opaque switching. In order to become mating competent *C. albicans* cells have to become MTL homozygous (a/a or α/α) [41], [42]. For this to happen there must be alterations at the chromosomal level, such as gene conversion, crossing-over events or loss of one copy (LOH) of chromosome 5, where MTL gene is located, and subsequent duplication of the remaining copy [42].

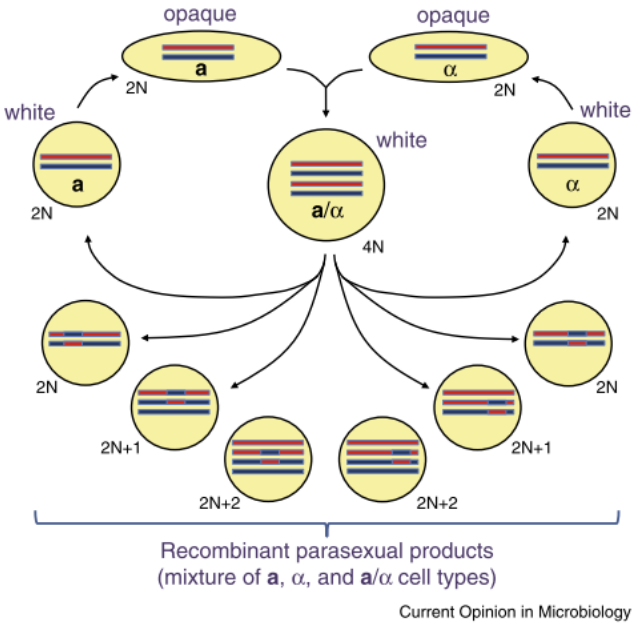

Figure 4. Genetic rearrangements during the parasexual cycle in *C. albicans*. Adapted from *Bennett RJ. The parasexual lifestyle of Candida albicans. Curr Opin Microbiol 2015;*

Then, the MTL homozygous white cells have to switch to the opaque state for mating to occur and subsequently form tetraploid (4N) a/α cells. Instead of go through conventional meiosis, tetraploid mating products return to a diploid (2N), or near-diploid state, by a parasexual mechanism of concerted chromosome loss accompanied by genetic recombination that origins a large number of aneuploid cells (e.g., 2N + 1 and 2N + 2 cells) (Figure 4) [40]. The existence of semi-stable, non-diploid *C. albicans* cell types highlights the flexibility of the *C. albicans* genome. Recently, stable haploid cells have also been detected [43].

1.4.1 Epidemiology

Candida albicans is the most common invasive fungal pathogen of humans, accounting for approximately 400,000 life-threatening infections per year and a far greater number of mucosal infections [44]. It is one of the most common cause of superficial vaginal or mucosal oral infections and may also, under propitious conditions, enter the bloodstream and lead to systemic infections that are life threatening, reaching mortality rates around 40% [30].

The risk of developing systemic candidiasis is higher in pregnant, diabetic, elderly or immunocompromised individuals like HIV-positive patients or organ transplant recipients taking immunosuppressive drugs, or in those who wear dentures or are receiving broad-spectrum antibiotic or corticosteroid treatment [31], [45], [46]. Although *C. albicans* is the main responsible for invasive candidiasis (46.3%), there are other species that can cause infection such as *C. glabrata* (24.4%) and *C. parapsilosis* (8.1%) [30].

Despite the advance in diagnosis and antifungal therapies, these infections are still responsible for high mortality rates. This may be related to the host status, but also rely on other factors such as diagnostic inadequacies, increasing drug resistance and small number of antifungals available for clinical use [47]. To further complicate the problem, persistent candidemia is often identified (continued isolation of the same *Candida* species in the blood of a candidemic patient) but its mechanisms are still poorly understood.

1.4.2 Pathogenicity

One of the main characteristics that establish *C. albicans* as a successful pathogen is its huge adaptability, giving them the ability to grow on the most diverse microenvironments in the host niches with different nutrient availability, pH, hypoxia and CO_2 levels. To infect a diverse range of host niches, *C. albicans* requires a wide range of virulence factors and fitness attributes [48], [49].

1.4.2.1 METABOLIC FLEXIBILITY

Unlike many other commensal microbes, that require specific carbon sources in order to proliferate (e.g. glucose), *C. albicans* has the capacity of metabolizing a diversity of carbon sources present in different host niches, such as sugars, fatty acids, amino acids and short chain carboxylic acids (lactate) by differential activation of glycolysis,

glyconeogenesis or the glyoxylate cycle. Besides that, *C. albicans* is also able to mount a strong stress responses and can survive periods of starvation, providing it the fitness attributes that are crucial for its survival in the hostile environment of the host [49]–[51].

Some studies have shown that *C. albicans* can assimilate glucose as well as alternative carbon sources at the same time, contributing to its metabolic flexibility and virulence [49].

1.4.2.2 BIOFILM FORMATION

Most microorganisms naturally form biofilms during their growth, creating densely packed communities of cells adhered to a surface. A major virulence attribute of *Candida albicans* is its ability to form morphologically complex biofilms composed of multiple cell types (round budding yeast-form cells, oval pseudohyphal cells, and elongated hyphal cells) [51].

In addition to that, *C. albicans* is able to create biofilms either on biotic surfaces, such as oral and vaginal epithelia, and abiotic surfaces, like implanted medical devices. Since biofilms have an increased resistance to chemical and physical injury, diverse stresses and host immune defence mechanisms, they are very difficult to combat in clinical settings [51], [52].

Biofilm formation occurs in five sequential steps: adherence of the round yeast cells to a surface, initial formation of colonies to form a basal layer, secretion of extracellular polymeric substances, maturation in a three-dimensional structure with proliferation of hyphal cells and cell dispersion [52].

1.4.2.3 ADHESION CHARACTERISTICS

After the first contact with the host cells, a series of interactions will take place between the host epithelial surface receptors and the specific adhesins expressed by *C. albicans,* leading to the attachment and the invasion to the host cells [53].

The most well-known *C. albicans* adhesins are the ALS (Agglutinin-Like Sequence) family and the Hwp1p (Hyphal Wall Protein 1). The eight ALS genes (ALS1–7 and ALS9) encode for large glycoproteins that are glycosylphosphatidylinositol-linked (GPI-linked) to the β-1,6-glucans of the fungal cell wall [53]. Hwp1p (Hyphal Wall Protein 1) is also

expressed as a GPI-linked protein interacting with host proteins, resulting in covalent attachment to the host epithelial cells [53].

Some of these adhesins are mainly expressed during hypha formation, namely Als3 and Hwp1, making this morphology particularly adherent. [53], [54].

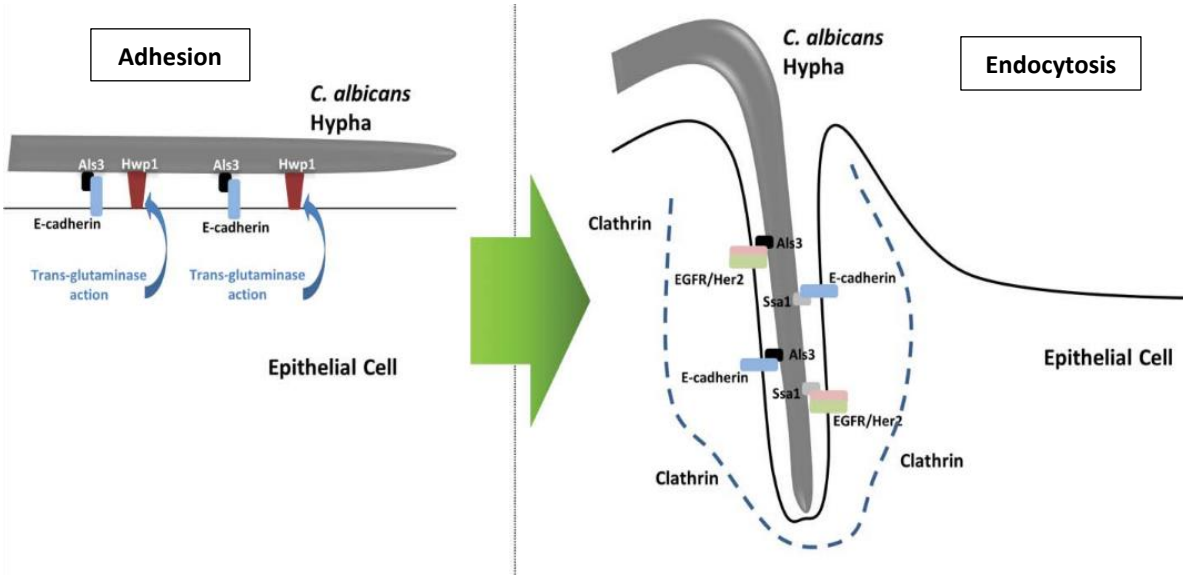

Figure 5. Adhesion and endocytosis of Candida albicans hyphae by epithelial cells. Adapted from *D. L. Moyes et al., "Candida albicans- epithelial interactions and pathogenicity mechanisms: scratching the surface," vol. 5594, no. October 2016, pp. 338–346, 2015.*

C. albicans hyphae cells have the capacity of interact with cell surface receptors and induce its endocytic uptake, in this case, by epithelial cells, in two stages. In the first one, adhesins such as Alps3p bind to their target cellular receptors (E-cadherin) or bind covalently to the cell surface (Hwp1p and host transglutaminases). On a second stage the *C. albicans* invasins Als3p and Ssa1p interact with target host receptors, E-cadherin and the EGFR/Her2 heterodimer, triggering activation of these receptors. This will lead to the induction of endocytosis via recruitment of clathrin and cytoskeletal reorganisation to form an invasion pocket down which the hypha invades into the host cell (Figure 5) [53].

1.4.2.4 SECRETED HYDROLASES

C. albicans hyphae cells are able to secrete different types of hydrolases (proteases, phospholipases and lipases), which have been proposed to facilitate active penetration into host cells and contribute to virulence. The most well studied are the proteases, necessary for the degradation of tissues, more specifically, the secreted aspartic proteases family (Saps) [37], [55].

1.5 Antifungal drugs and resistance

1.5.1 Mechanisms of action of antifungal drugs

The reason why the range of antifungal drug classes and drug targets is limited is the evolutionary similarity between fungal and human cells. The currently available antifungal drugs for clinical use can be included in the following classes: azoles, echinocandins, polyenes and nucleoside analogues [56], [57].

Most of the antifungal drugs act at the level of ergosterol biosynthesis, which is the main sterol on the membrane of fungal cells, unlike the mammal cells, in which it is cholesterol. Amphotericin B was one of the most used drug in treatment of fungal infections for years, however it has fallen into disuse because of its toxicity and interaction with cholesterol present on the host-cell membranes [47].

The members of the azole class of antifungal drugs, and fluconazole in particular, have been the drug of choice for the treatment of invasive fungal infection for the last decades, because they are affordable, accessible and because of its oral bioavailability [56].

1.5.1.1 AZOLES

Azoles are the most common class of antifungal used and can be divided into two categories – Imidazoles and Triazoles – differing on the number of nitrogen atoms in the azole ring (two for Imidazoles and three for Triazoles). Imidazole antifungals include Clotrimazole, Econazole, Miconazole, Ketoconazole and Tioconazole whereas Triazoles include Fluconazole, Itraconazole, Posaconazole and Voriconazole. This type of antifungal

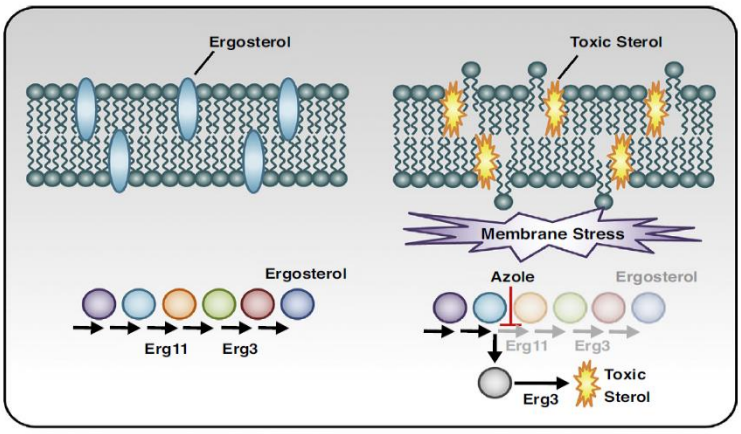

Figure 6. The azoles act by targeting the ergosterol biosynthetic enzyme lanosterol demethylase, encoded by ERG11, leading to the accumulation of toxic sterol. Adapted from *N. M. Revie et al. "Antifungal drug resistance: evolution, mechanisms and impact,"* Curr. Opin. Microbiol., *vol. 45, pp. 70–76, 2018*

drugs interact with the membrane of fungal cells, in particular affecting the biosynthesis of ergosterol [47], [58].

Triazoles inhibit the ergosterol biosynthetic pathway by targeting the cytochrome P450-dependent enzyme lanosterol 14-α demethylase, encoded by ERG11 in yeast. This results in an accumulation of toxic sterol intermediates produced by ERG3, that depletes the ergosterol from fungal cell membrane, disturbing the membrane stability and suppressing the fungal growth (Figure 6) [59].

1.5.1.2 POLYENES

The target of the polyenes, including amphotericin B and nystatin, are also plasma membrane sterols, more specifically ergosterol. Their mechanism of action involves the formation of concentration-dependent channels that lead to the cell dead by allowing ions like potassium and other essential cytoplasmatic materials to escape. Another mechanism of action of amphotericin B is the formation of extramembranous aggregates that extract ergosterol from lipid bilayers (Figure 7) [60], [61].

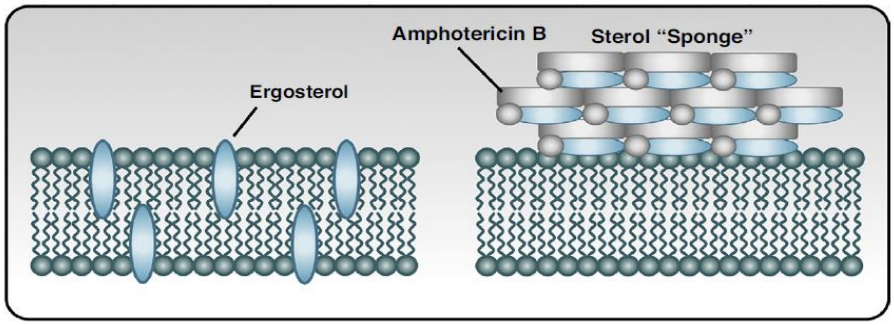

Figure 7. Mode of action of the polyenes such as Amphotericin B. Adapted from *N. M. Revie et al. "Antifungal drug resistance: evolution, mechanisms and impact,"* Curr. Opin. Microbiol., *vol. 45, pp. 70–76, 2018.*

1.5.1.3 ECHINOCANDINS

The echinocandins drugs, such as anidulafungin, caspofungin, and micafungin, are lipopeptides and the most recent class of antifungal drugs released into the clinic. In current guidelines, they are the recommended first-line therapy for candidemia, due to their clinical efficacy, fungicidal activity, favourable safety profile, limited drug interactions, and concerns about azole resistance. They compromise the fungal cell wall synthesis by inhibiting (1,3)-β-D-glucan synthase (a key enzyme involved on the synthesis of the structural polymer β-1,3-glycan, encoded by FKS1 (Figure 8) [58], [59], [61], [62].

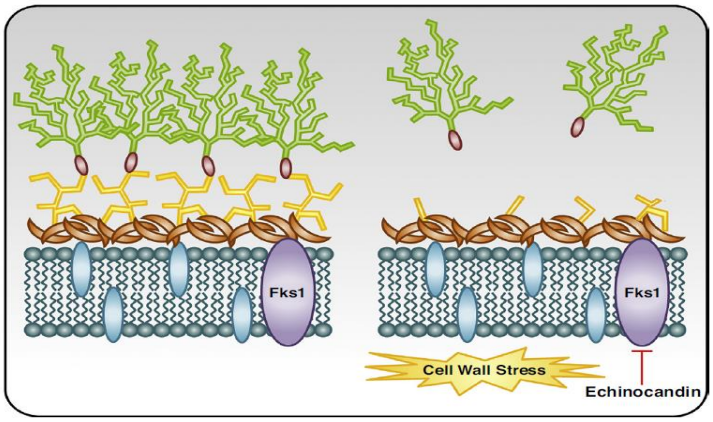

Figure 8. Echinocandins mode of action: inhibition of (1,3)-β-D-glucan synthase, leading to loss of cell wall integrity. Adapted from *N. M. Revie et al. "Antifungal drug resistance: evolution, mechanisms and impact,"* Curr. Opin. Microbiol.*, vol. 45, pp. 70–76, 2018*

1.5.1.4 NUCLEOSIDE ANALOGUES

Flucytosine (5-FC) is a pyrimidine analogue with fungistatic properties that uses cytosine permeases to enter the fungal cell and inhibits the thymidylate-synthetase enzyme interfering with DNA. 5-FC can also be converted to 5-fluorouracil which in turn will interact with RNA [57].

5- FC is rarely used alone for the treatment of candidiasis, since it induces side-effects, such as hepatic impairment, interference with bone marrow function, and rapid development of resistance especially among *Candida* species [63].

1.5.2 Mechanisms of drug resistance

The most commonly used antifungal drugs, including fluconazole, are fungistatic, which means that they only stop the fungi from growing instead of killing them. This gives them time to, under pressure, develop mechanisms to become more resistant, like becoming able to rapidly replace the fungal proteins the drug destroys or to efficiently remove the drug from its cells [64].

1.5.2.1 RESISTANCE TO AZOLES

Some of the mechanisms leading to azole resistance are point mutations occurring on the ERG11 gene (which encodes the target protein) that decrease the binding affinity between the protein and the drug; the increased activity of ergosterol pathway enzymes (ERG11) via gain-of-function mutations in the transcriptional activator Upc2, leading to an increment of the intracellular concentration of the target protein and consequently to the reduction of the drug impact; the overexpression of efflux membrane ATP-binding cassette transporters (encoded by CDR1 and CDR2) that has been reported as the most common cause of azole resistance in *C.albicans* and the major facilitator superfamily efflux pump (encoded by MDR1), reducing the intracellular drug concentration; biofilm formation may cause resistance to antifungal drugs as well [60], [64]–[66].

Other alterations such as loss of function of the Δ-5,6- desaturase enzyme Erg3, can also lead to azole resistance. ERG3-mediated azole resistance depends on key stress response regulators such as the protein phosphatase calcineurin, the protein kinase Pkc1 and the molecular chaperone Hsp90 (Figure 9) [59].

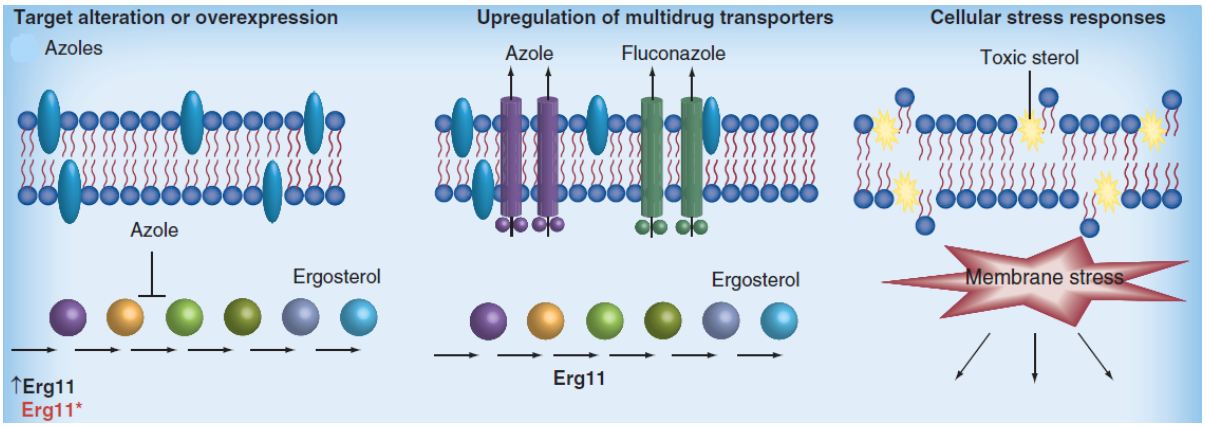

Figure 9. Mechanisms of azole resistance include overexpression or alteration of the drug target, overexpression of multidrug transporters, or cellular alterations that mitigate drug toxicity or enable responses to drug-induced stress. The coloured circles represent intermediates in ergosterol biosynthesis. Adapted from *J. L. Xie, E. J. Polvi, T. Shekhar-guturja, and L. E. Cowen, "Elucidating drug resistance in human fungal pathogens," vol. 9, pp. 523–542, 2014.*

Ford *et al.* found that the loss of genetic information of one of the copies from genes encoding surface components or involved in expelling drugs from the cell, is linked to resistance to fluconazole [64].

1.5.2.2 RESISTANCE TO POLYENES

Resistance to polyenes has been documented in clinical *Candida* isolates from patients receiving either polyene or azole treatment and is associated with mutations in genes encoding components of the ergosterol biosynthesis pathway [67]. Amphotericin B, one of the most common antifungals from the polyene family, is typically fungicidal, which means that the development of resistance is associated with the selection of the less susceptible species during therapy. The mechanism of resistance to amphotericin B involves a reduction in ergosterol content in the cell membrane. For example, a previous treatment with an azole antifungal can decrease the presence of sterol in the membrane and if it is followed by a polyene treatment it could trigger resistance [61]. Indeed, cross-resistance to amphotericin B has been identified in azole-resistant *C. albicans* clinical isolates containing a defective sterol Δ-5,6 desaturase (Erg3) [67].

1.5.2.3 RESISTANCE TO ECHINOCANDINS

Although echinocandins have been used in the clinic for only a relatively short time, cases of resistant isolates from patients treated with this antifungal have been documented. Several mechanisms of resistance to echinocandins have been reported , such as alteration of the drug target, upregulation of multidrug transporter or cellular stress responses [68].

The mechanism of echinocandin resistance in *Candida* species involves genetic acquisition of mutations in FKS genes, which encode the catalytic subunits of glucan synthase [61].

1.6 Population heterogeneity and drug tolerance

Heterogeneity may cause drug tolerant subpopulations of cells to exist within a population. The appreciation of different responses and phenotypes within a genetically identical population of cells, represents a recent paradigm shift in the field of microbiology. This small proportion of cells could be important for the overall success of drug therapy.

A recent work showed that tolerance is due to the slow growth of subpopulations of cells that overcome drug stress more efficiently than the rest of the population, and correlates inversely with intracellular drug accumulation. The distinction between resistance and tolerance is consistent with the mechanisms that impact them: resistance mechanisms directly affect the drug target or its concentration in the cell, thereby enabling efficient growth in the presence of the drug. By contrast, tolerance reflects stress response strategies that are indirect and may enable survival despite the continued ability of the drug to interact with its target, to remain in the cell (albeit at lower average concentrations) and to affect cell growth [69].

Drug tolerant subpopulations of cells, named persisters, have been identified within *Candida* biofilms. These cells survive in the presence of high concentrations of lethal antifungals, but are not resistant since they do not grow in the presence of antimicrobials, harbor stable genetic mutations, or have an increased the minimum inhibitory concentration (MIC) [70].

1.7 Objectives

Mistranslation in *C. albicans* produces advantageous phenotypic variability, including increased fitness in the presence of fluconazole. The underlying molecular mechanism is not understood, but this work aims to test the hypothesis that mistranslation produces subpopulations of *C. albicans* cells that are reservoirs of drug tolerance from where resistance may emerge.

The specific objectives of this thesis are the following:

1. Carry out *in vitro* evolution studies to clarify whether prolonged antifungal therapy impacts mistranslation;

2. Determine whether the population segregates into “hypermistranslator” and relatively “wild-type” cell types upon challenge with fluconazole;

3. Carry out direct competition experiments between wild type and hypermistranslating cells and measure the relative survival of each during the course of the evolution experiment;

4. Assess tolerance and/or resistance to antifungals during evolution.

2 Material and Methods

2.1 Strains and growth conditions

The *C. albicans* strains used in this study T0, T1, T1KO1, T2, T2KO1 and T2KO2 were engineered by Bezerra and colleagues, and exhibit different levels of leucine misincorporation at serine CUG codons [27] (Table 1).

The strains were grown at 30ºC in Yeast Peptone Dextrose (YPD), containing 2% glucose, 1% yeast extract and 1 % peptone, unless otherwise specified.

Table 1. *C.albicans* strains used in this study.

Strains	Genotype	% Leu incorporation
T0	arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ ura3Δ::imm434/ura3Δ::imm434 iro1Δ::imm434/iro1Δ::imm434 RPS1/rps1Δ::pUA709 (URA3)	1,45
T1	arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ ura3Δ::imm434/ura3Δ::imm434 iro1Δ::imm434/iro1Δ::imm434 RPS1/rps1Δ::pUA702 (URA3, Sc tLCAG)	20,61
T2	arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ ura3Δ::imm434/ura3Δ::imm434 iro1Δ::imm434/iro1Δ::imm434 RPS1/rps1Δ::pUA706 (URA3, Sc tLCAG, Sc tLCAG)	67,29
T1KO1	arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ ura3Δ::imm434/ura3Δ::imm434 iro1Δ::imm434/iro1Δ::imm434 RPS1/rps1Δ::pUA702 (URA3, Sc tLCAG) tSCAG/tscagΔ::ARG4	50,04
T2KO1	arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ ura3Δ::imm434/ura3Δ::imm434 iro1Δ::imm434/iro1Δ::imm434 RPS1/rps1Δ::pUA706 (URA3, Sc tLCAG, Sc tLCAG) tSCAG/tscagΔ::ARG4	80,84
T2KO2	arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ ura3Δ::imm434/ura3Δ::imm434 iro1Δ::imm434/iro1Δ::imm434 RPS1/rps1Δ::pUA706 (URA3, Sc tLCAG, Sc tLCAG) tscagΔ::ARG4/tscagΔ::HIS1	98,46

2.2 Laboratory evolution experiments

2.2.1 Evolution of control T0 strain

9 clones of the control strain T0 were inoculated in RPMI 1× media (Sigma) in a 96-well plate and incubated at 37ºC.. An aliquot of stationary phase cultures was transferred to fresh media every 2 days until approximately 200 generations. T0 was evolved in two different conditions: without drug and in the presence of fluconazole (0,125 µg/ml).

The levels of mistranslation during the evolution of T0 in both conditions were assessed using a reporter system based on the yeast enhanced green fluorescent protein

(yEGFP) described by Bezerra and colleagues [27]. This reporter only becomes active if mistranslation occurs, and the GFP fluorescence allows the detection/quantification of mistranslation by flow cytometry (section 2.3).

2.2.2 Pairwise competition evolution

The control strain T0 was tagged with mCherry while the high mistranslating strains (T1, T1KO1, T2, T2KO1 and T2KO2) were tagged with GFP which allowed strain differentiation by detection of cell fluorescence. Cells expressing each fluorescent protein were mixed together in approximately equal numbers (2×10^5 CFU/ml) (as confirmed by flow cytometry) at the start of the evolution experiments in YPD with or without fluconazole. The strains were combined in competition pairs as shown in Table 2.

Table 2. Competition pairs used in this study.

I	T0 vs. T1
II	T0 vs. T1KO1
III	T0 vs. T2
IV	T0 vs. T2KO1
V	T0 vs. T2KO2

3 replicates of each competition I, II, III, IV and V (described in table 2) were inoculated in YPD media without drug (row A) and rows C, E and G were inoculated with increasing concentrations of fluconazole (C-0,25 µg/ml, E-1µg/ml and G-4 µg/ml) in a 96-well format plate and incubated at 37ºC (Figure 10) . An aliquot of stationary phase cultures was transferred to fresh YPD media (with the same concentrations of fluconazole) every 2 days until approximately 400 generations. All competitions from each passage were frozen with 40% glycerol at -80ºC.

The relative proportions of the 2 subpopulations (GFP, mCherry) were measured using a flow cytometer BD Accuri C6 (BD Biosciences, San Jose, CA) (section 2.6).

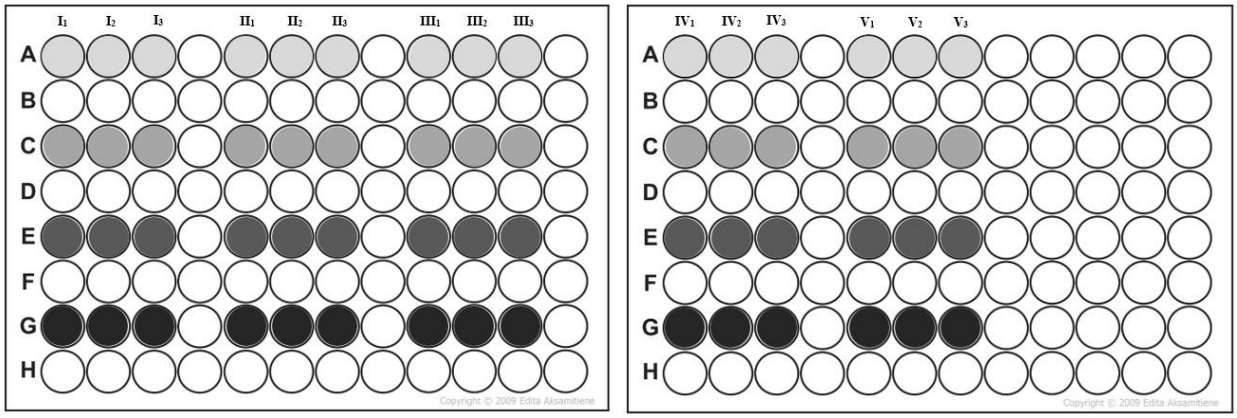

Figure 10. 96-well plate used in the evolution competition with fluconazole. The I, II, III, IV and V correspond to the competitions listed on table 2 used in triplicate. They were grown in two different conditions: YPD liquid media without drug and in the presence of different concentrations of fluconazole of 0,25 µg/ml, 1 µg/ml and 4 µg/ml, corresponding to the different rows A, C, E and G, respectively.

2.3 Quantification of mistranslation

The levels of mistranslation during the evolution were assessed using a reporter system based on the yeast enhanced green fluorescent protein (yEGFP). This gain-of-function reporter system was developed by Bezerra and colleagues and only become active (expresses functional GFP) if Leu is incorporated at codon 201 (Leu_{201}). If serine is incorporated at the same site, it will lead to a destabilization and rapid degradation of GFP [27].

The Leu UUA codon at position 201 is a positive control – always incorporates leucine and always emits fluorescence, the Ser UCU codon is negative control - always incorporate serine, which destabilizes GFP, and the ambiguous CUG codon which can

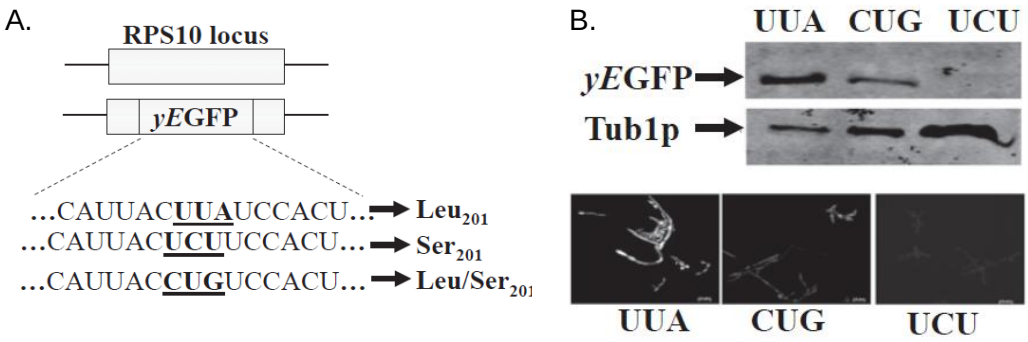

Figure 11. Fluorescent reporter system to quantify leucine insertion at CUG positions *in vivo*. (A). The system is based on the yEGFP gene. The Leu UUA codon at position 201 was mutated to the ambiguous CUG codon and a UCU Ser codon. Incorporation of Ser at this position inactivated yEGFP, whereas Leu misincorporation at the CUG position provided a functional fluorescent protein. **(B).** Western blot analysis of the three versions of the reporter. No band was detected when Ser was inserted at position 201. Tubulin was used as an internal control. Adapted from A. R. Bezerra et al. "Reversion of a fungal genetic code alteration links proteome instability with genomic and phenotypic diversification," Proc. Natl. Acad. Sci., 2013.

incorporate both leucine and serine. This will allow the quantification of mistranslation rate once the fluorescence emitted will be directly proportional to the insertion of leucine at that specific position (Figure 11).

The degree of activity detected (intensity of fluorescence) allows the quantification of the mistranslation rate by flow cytometry when compared to the wild-type functional protein.

yEGFP expression was quantified using flow cytometry at the beginning and at the end of the experimental evolution both with drug and without drug. T0 cells were grown overnight in liquid medium to an optical density at 600 nm of 2,0-2,5 and aliquots were analysed by flow cytometry using the flow cytometer BD Accuri C6 (BD Biosciences, San Jose, CA) (section 2.6).

Mean fluorescence intensities (± standard deviation) were quantified in individual *C. albicans* cells containing the reporter yEGFP Leu-UUA_{201} (positive control), Ser-UCU_{201} (negative control) and Ser/Leu -CUG_{201} (reporter).

2.4 Determination of broth dilution MICs

In order to test the susceptibility to fluconazole of the populations in each competition, we used a microdilution method based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST protocol) with alterations. The method is based on the preparation of working solutions of antifungal agents in 100 µl volumes/well (with the addition of an inoculum also in a volume of 100 µl). Standardisation of the inoculum is crucial to achieve accurate and reproducible antifungal susceptibility tests. The test was performed in plates of 96 wells and the range of concentrations of fluconazole used varied from 0 µg/ml to 6 µg/ml (Figure 12). Each well was inoculated with 100 µl of 1×10^5 CFU/ml yeast suspension (cells were counted using the TC10^{tm} Automated cell counter from BioRad) from each competition (I, II, III, IV and V). The microdilution plates were incubated without agitation at 37ºC for 24h and then, the absorbance was read at 595 nm using the iMARK^{tm} Microplate Reader.

If the measured absorbance was less than 0,2 after the 48h of incubation it was considered a failed test. The value of the MIC is defined by the lowest concentration of fluconazole necessary to inhibit the growth of 50% of the population when compared with the drug free control.

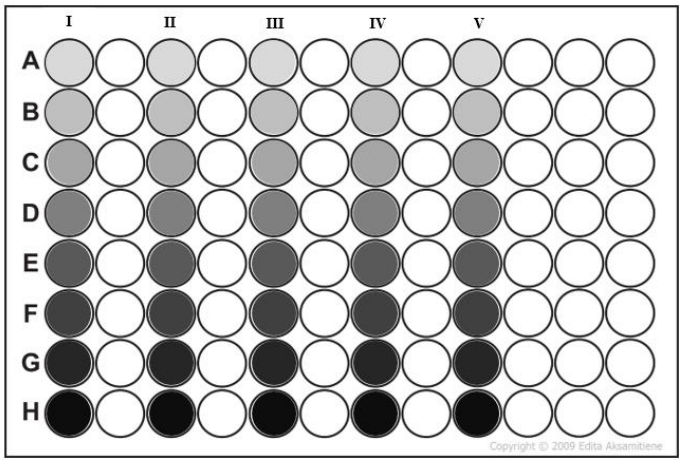

Figure 12. 96-well plate used to determine broth MICs. One clone from each competition (I, II, III, IV and V) was inoculated according with the representation on the scheme. Rows A, B, C, D, E, F, G and H correspond to different and crescent fluconazole concentrations (A – 0 µg/ml; B- 0,125 µg/ml; C- 0,250 µg/ml; D- 0,500 µg/ml; E-0,750 µg/ml; F- 1,5 µg/ml; G- 3 µg/ml; H- 6 µg/ml).

2.5 Antifungal susceptibility testing (E-test)

For the E-tests, strains were grown in synthetic defined (SD) medium without uracil containing 0,67% yeast nitrogen base, 2% glucose, and 0,2% dropout mix (with 2% agar for solid medium only) at 30ºC. Cells from each competition from both initial and final passages were grown to mid-log phase, washed in phosphate-buffered saline 1× (PBS 1×), and diluted to an OD_{600} of 0,015. 150 µl of cells were plated on SD minus uracil agar plates (pH 7) with glass beads and allowed to dry for 15 to 30 min before a fluconazole E-test strip (0,016 to 256 µg/ml; AB Biodisk) was applied. Plates were incubated at 30°C for 48 h, and the MIC was determined as the concentration at which the first growth inhibition ellipse occurred [71].

2.6 Flow cytometry

Flow cytometry was used to distinguish the different populations (control strain (T0) vs. hypermistranslating strain) within one competition and to calculate the ratio of control/mistranslator cells by monitoring GFP and mCherry fluorescence.

The degree of misincorporation of leucine (mistranslation) was also quantified by flow cytometry in cells carrying the reporter described in section 2.3. In both cases the Accuri C6 flow cytometer equipped with an autosampler (BD) was used.

Cells were grown overnight on 96 well plates at 37ºC and were filtered before the analysis using the Sterile Cell Strainer from Fisher brand (40 µm Nylon Mesh).

Analysis was based on light-scatter and fluorescence signals. Signals corresponding to forward angle and 90º-side scatter (FSC and SSC) and fluorescence were accumulated, the fluorescence signals (pulse area measurements) being screened by the following filter configurations: FL1: a 530/30 nm band-pass filter for GFP. A total of 10 000 events were recorded at a slow flow rate setting (14 $\mu L_{sample/min}$). The acquired data were analysed in the Accuri C6 Sampler software.

2.7 Fluorescence microscopy

Fluorescence microscopy was used to distinguish the cells tagged with mCherry from the cells tagged with GFP.

Fluorescence was detected using a Zeiss MC80 Axioplan 2 light microscope, equipped for epifluorescence microscopy with the filter set HE38. Photographs were taken using an AxioCam HRc camera and images were analysed using ImageJ software.

3 Results

3.1 Prolonged antifungal therapy impacts mistranslation

C. albicans has the interesting capacity of supporting high levels of mistranslation. Under regular environmental conditions, the CUG codon is decoded as serine 97% of the times and leucine 3% of the times and this ambiguity generates phenotypic diversity. Under stress conditions, levels of leucine misincorporation are variable and tend to increase, as already shown by the work of Gomes *et al.* Per example, for cells growing at 30ºC the level of mistranslation is 2,96% whereas at 37ºC the value increases to 3,9%, and the same happens when growth under stress conditions like low pH (4,95%) and in the presence of H_2O_2 (4,03%) [11].

The ability of *C. albicans* to tolerate high levels of mistranslation was also confirmed on the study made by Bezerra and colleagues by showing that the CUG identity was almost completely reverted from Ser back to Leu (98.46% in strain T2KO2). They discovered that ambiguous cells are tolerant to commonly used antifungals, namely fluconazole, indicating that CUG ambiguity may be relevant to evolution of antifungal drug resistance [27]. Recently, the host laboratory showed that mistranslation accelerates the acquisition of resistance to fluconazole but alterations in ambiguity levels during experimental evolution was not performed [71].

In order to evaluate whether prolonged antifungal therapy impacts mistranslation we measured the global mistranslation rate of *C. albicans* cells cultivated over extended periods of time in the presence and the absence of fluconazole. We also determined whether the population segregates into “hypermistranslator” and relatively “wild-type” cell types upon challenge with environmental conditions known to increase mistranslation (fluconazole).

To do so, 9 clones of strain T0 carrying the fluorescent reporter described in section 2.3 were evolved: 3 were positive controls (UAA_{201} codon), 3 were negative controls (UCU_{201} codon) and 3 were reporter clones (CUG_{201} codon).

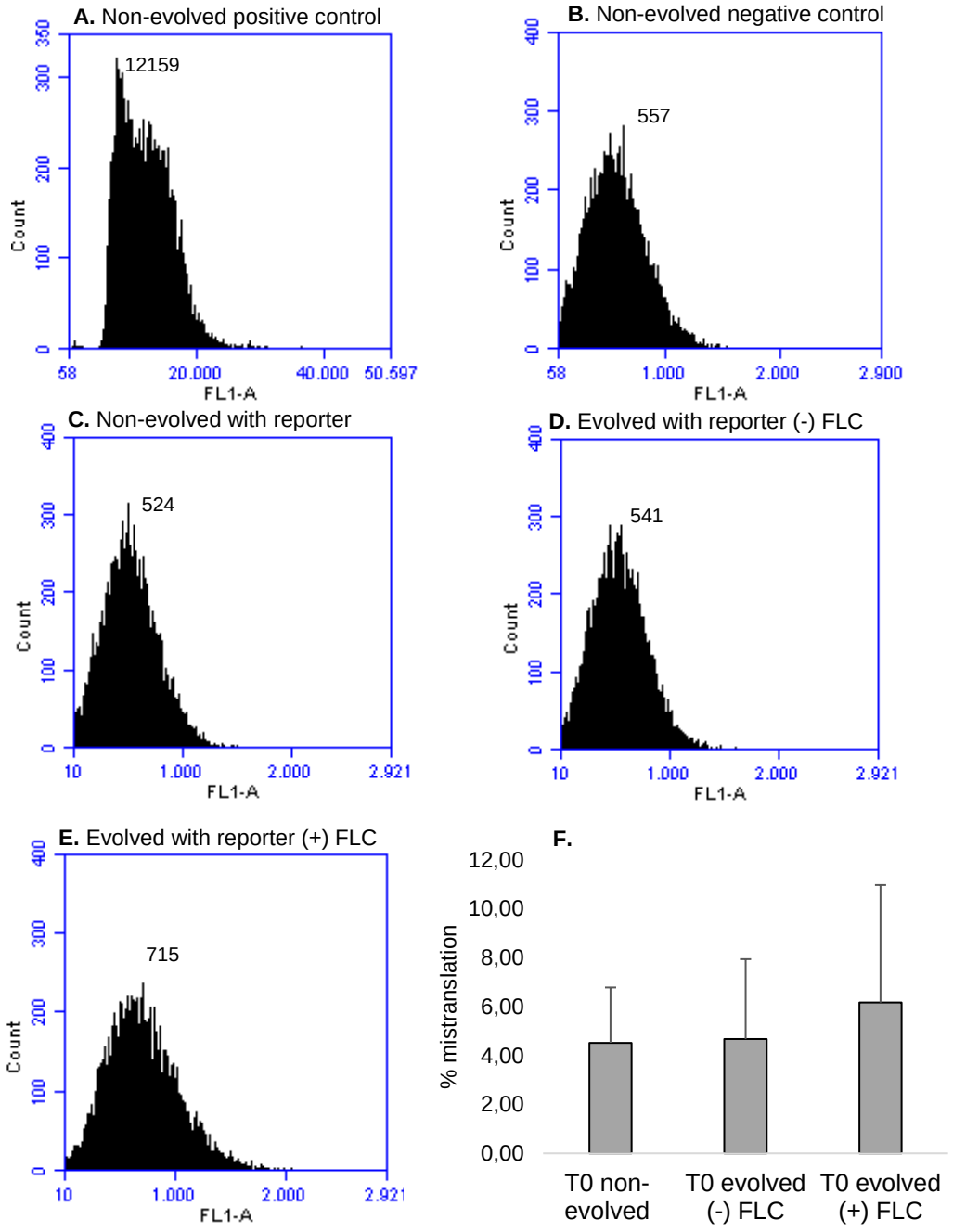

Figure 13. GFP fluorescence intensity (FL1-A) in T0 cells carrying the mistranslation reporter was monitored by flow cytometry at the beginning and end of the evolution. In each time-point,10 000 cells were analysed by flow cytometry. **(A).** Non-evolved T0 positive control (UAA_{201} codon)**; (B).** Non-evolved T0 negative control (UCU_{201} codon); **(C).** Non-evolved T0 containing the reporter (CUG_{201} codon)**; (D).** T0 containing the reporter (CUG_{201} codon) evolved for 200 generations with no drug (- FLC) resulted in no alteration when compared with the beginning of the evolution; **(E).** T0 containing the reporter (CUG_{201} codon) evolved for 200 generations with fluconazole (+ FLC) **(F).** Percentage of mistranslation of non-evolved strain T0 and T0 evolved with no drug and with FLC. In the initial passage, the percentage of mistranslation was 4,52 ± 2,26. In the final passage, it was 4,67±3,27 and 6,17±4,81 referent to the evolution on media without drug and with drug, respectively. 3 replicates were tested and the statistical test, t-test, showed that there is no statistical significance (p=0,6202).

T0 clones were grown in liquid RPMI media without drug and in liquid media with a fluconazole concentration of 0,125 µg/ml and inoculated in a 96-well plate. Then, every 2 days, passages were done, until about 200 generations. In the end of the evolution, the intensity of fluorescence was analysed by flow cytometry. Figures 13A, 13B and 13C show examples of the fluorescence intensity of non-evolved T0 positive control (UAA_{201} codon), T0 negative control (UCU_{201} codon) and T0 reporter clone (CUG_{201} codon), respectively. Figure 13D shows fluorescence intensity of evolved T0 reporter (CUG_{201} codon) without drug, which is very similar to the profile in Figure 13C. Figure 13E shows fluorescence intensity of evolved T0 reporter (CUG_{201} codon) in the presence of fluconazole. After normalization of fluorescence intensity levels, values of mistranslation were calculated.

The results presented in the Figure 13 F show that the values of mistranslation in T0 does not have significant alterations over the entire evolution experiment. In the beginning of the evolution, the percentage of mistranslation was 4,52 ± 2,26 % and it remains similar in the final passage without fluconazole 4,67±3,27%. In the end of evolution with fluconazole, the value slightly increased to 6,17±4,81%, nevertheless, these results do not have statistical significance (Figure 13 F.). Therefore, we can conclude that in the context of the global population there is no change in mistranslation levels during evolution with a constant concentration of fluconazole.

However, when we analysed the cytometry profile of evolved T0 reporter (CUG_{201} codon) in the presence of fluconazole with more detail (Figure 14 A.) a division into a

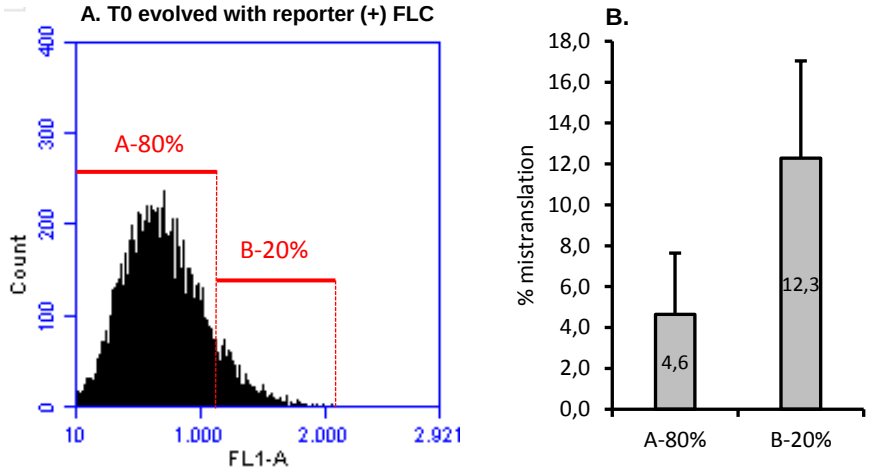

Figure 14. Distribution of the T0 population into sub-populations in the presence of fluconazole. (A). At the end of the evolution of the strain T0 with fluconazole there is an alteration in the distribution of cells compared with the initial population. Formation of a sub population of cells with an increased fluorescence –indicative of increased mistranslation rate (subpopulation B corresponds to 20% of cells of the population with fluorescence intensity above 1200). **(B).** Representative graphic of the levels of mistranslation on T0 strain cells after evolution with drug. Subpopulation A includes 80% of cells and its error rate was 4,6 ± 3,0%. Subpopulation B includes only 20% of the entire population and its error rate was 12,3 ± 4,76%.

subpopulation that shows a slight increase of fluorescence intensity. Although, the majority of the population (80% - subpopulation A) has a percentage of mistranslation of 4,6±3,0%, a small group of cells (20% - subpopulation B) showed levels of mistranslation of 12,3±4,76% (Figure 14 B).

In other words, even though the medium value showed by the general population is considered a “normal” value of mistranslation, we see that there is a part of the population (subpopulation) that after 200 generations of evolution with antifungal treatment has an increase of mistranslation rate. Since cells with increased error are more tolerant to the effect of the drug (as shown by previous works from the host laboratory) they can constitute a problem in the context of a prolonged antifungal treatment.

3.2 Antifungal therapy contributes to selection of hypermistranslators (competition)

In the present study, we tested the hypothesis that fluconazole therapy may select hypermistranslator cells that are efficient colonizers with high tolerance to antifungals. To do so, we evolved control (T0) and hypermistranslating strains of *C. albicans* in competition with each other (see table 2) in the absence and presence of fluconazole for approximately 400 generations.

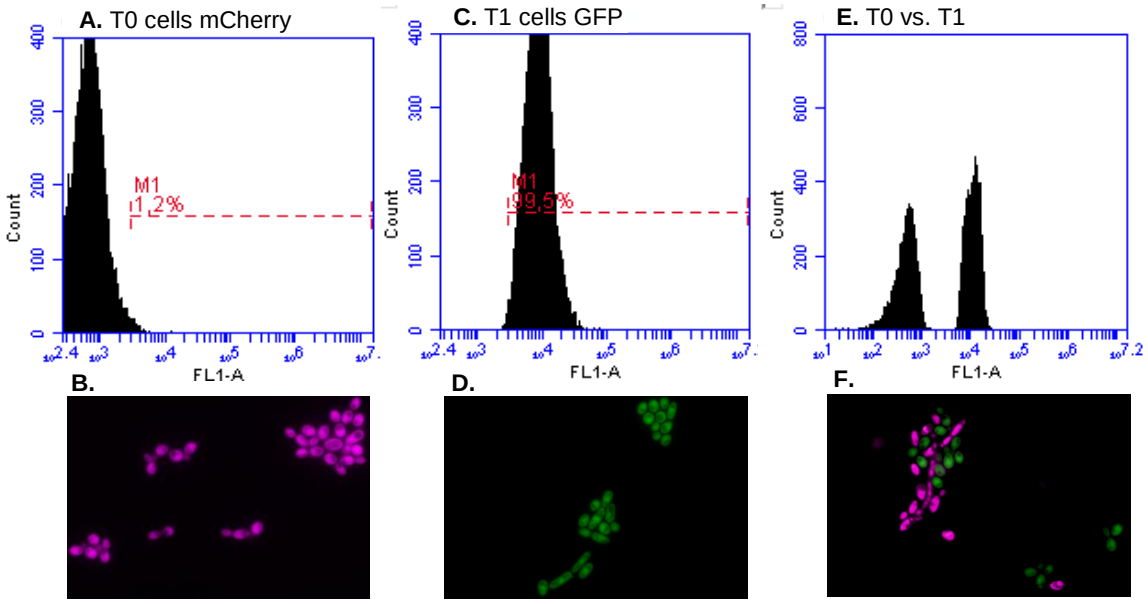

Figure 15. Fluorescence was monitored at the beginning of evolution on individual T0 cells tagged with mCherry. **(A)** and **(B),** hypermistranslating strain (T1 in this example) tagged with GFP **(C)** and **(D)** and T0 and T1 mixed together in a competition **(E)** and **(F)** by flow cytometry and by fluorescence microscopy.

The control strain (T0) was tagged with mCherry (Figure 15 A-B) while mistranslating cells were tagged with GFP (Figure 15 C-D), which allowed strain differentiation by flow cytometry and fluorescence microscopy as we can see in the example of Figure 15. Using the example of competition “T0 vs T1”, it is possible to differentiate between green cells (T1) and magenta cells (T0) within a population using the fluorescence microscopy image (figure 15 F.).

The histograms resulting from flow cytometry analysis showed that mCherry cells have a residual level of fluorescence using filter set FL1-A (GFP filter set) (Figure 15 A), while all T1 cells tagged with GFP have high levels of intensity (Figure 15 C). These controls allowed the establishment of region M1 as the region comprising cells with GFP.

When the 2 populations are mixed, we can clearly see a division between the two populations, one of them having higher levels of fluorescence (T1) while the other has residual levels of fluorescence (T0) (Figure 15 E.).

For all the competitions, at the beginning of the evolution experiment (20 generations), the population was divided in approximately 50 % of each competitor.

Figures 16 – 20 show the cytometry profiles and relative proportions of cells of each competition at the end of the evolution (400 generations). In all cases, in the absence of drug (Figures 16A, 17A, 18A, 19A and 20A), it is noticeable that only a small portion of the population (~20%) are within the M1 region, which means that approximately only 20% of cells are tagged with GFP at the end of evolution. The remaining cells are tagged with mCherry, meaning that at the end of the competition experiment without drug, T0 strain (control) relative fraction in the population is ~80% in every competition. This is not

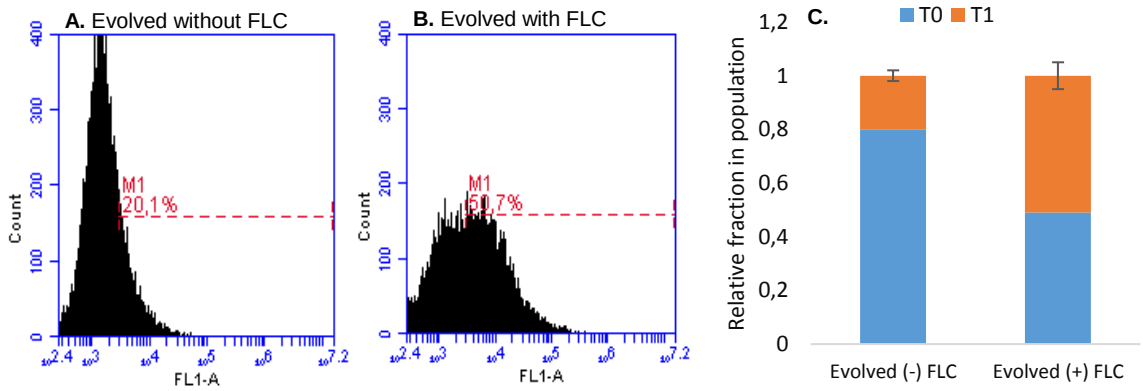

Figure 16. Fluorescence was monitored by flow cytometry at the end of the *in vitro* evolution experiment (400 generations) for competition T0 vs. T1 (A). End of the competition experiment without drug; **(B).** End of the competition experiment with drug ([fluconazole]= 4µg/ml); **(C).** Evolutionary dynamics of experimental populations in the end of the experiment (400 generations) in the absence (no drug) and presence of fluconazole (4µg/ml). The coloured bars represent the relative fractions of each subpopulation – control -strain T0 (blue), mistranslating strain T1 (orange)- as determined by flow cytometry.

surprising, considering the fact that T0 has a higher growth rate compared to the hypermistranslating strains [27] and in the absence of the stress caused by the drug they have advantage and take over the population.

On the other hand, at the end of competition experiments in the presence of drug (4 µg/ml of fluconazole) relative fractions of population have variations. On T0 vs. T1 (Figure 16) and T0 vs. T1KO1 (Figure 17) competitions the final population backs to levels similar to the beginning of the experiment. Approximately 51% of T0 vs. 49% of T1 and 57% of T0 vs. 43% of T1KO1.

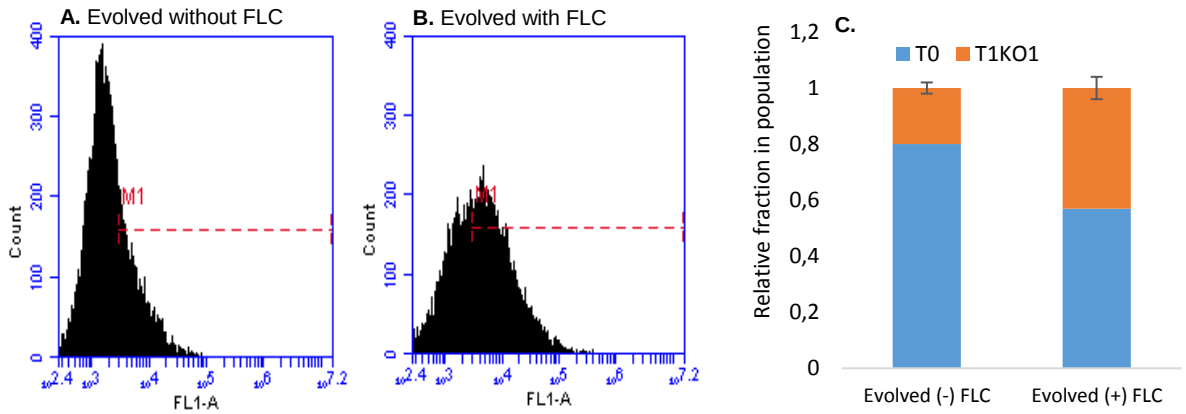

Figure 17. Fluorescence was monitored by flow cytometry at the end of the *in vitro* evolution experiment (400 generations) for competition T0 vs. T1KO1 (A). End of the competition experiment without drug; **(B).** End of the competition experiment with drug ([fluconazole]= 4µg/ml); **(C).** Evolutionary dynamics of experimental populations in the end of the experiment (400 generations) in the absence (no drug) and presence of fluconazole (4µg/ml). The coloured bars represent the relative fractions of each subpopulation – control -strain T0 (blue), mistranslating strain T1KO1 (orange)- as determined by flow cytometry.

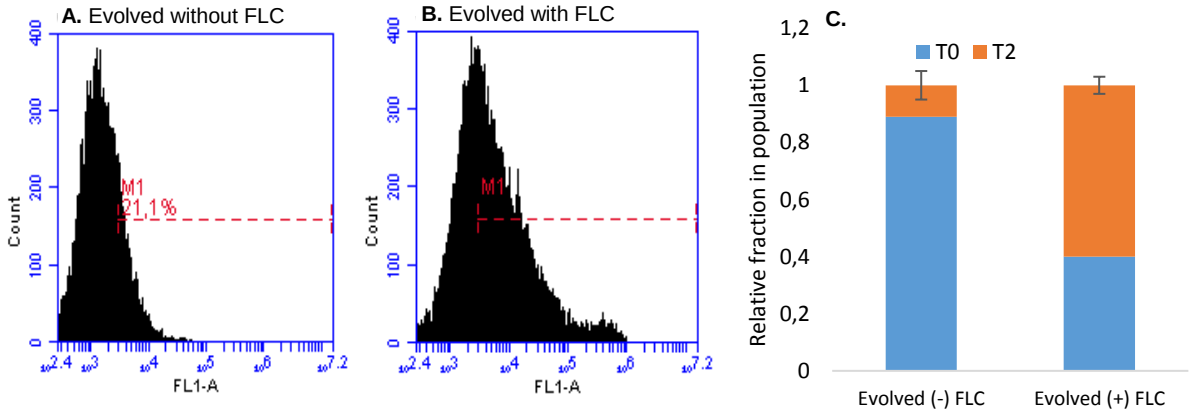

Figure 18. Fluorescence was monitored by flow cytometry at the end of the *in vitro* evolution experiment (400 generations) for competition T0 vs. T2 (A). End of the competition experiment without drug; **(B).** End of the competition experiment with drug ([fluconazole]= 4µg/ml); **(C).** Evolutionary dynamics of experimental populations in the end of the experiment (400 generations) in the absence (no drug) and presence of fluconazole (4µg/ml). The coloured bars represent the relative fractions of each subpopulation – control -strain T0 (blue), mistranslating strain T2 (orange)- as determined by flow cytometry.

On competition T0 vs. T2 (Figure 18) we start to see alterations, with T2 dominating the population with a relative fraction of 60±3% (figure 18 C.). On the remaining competitions, T0 vs. T2KO1 and T0 vs. T2KO2, the relative fractions of the hypermistranslating strain rises to 70±4% (figure 19 C.) and 78±3% (figure 20 C.) in the global population, respectively.

Altogether, these results demonstrate that there is a relation between the increased mistranslation levels and the proliferation of hypermistranslated cells within a competition. Antifungal therapy selected hypermistranslating cells and the impact of this selection on the tolerance/resistance phenotype of the population was studied in the next section of results.

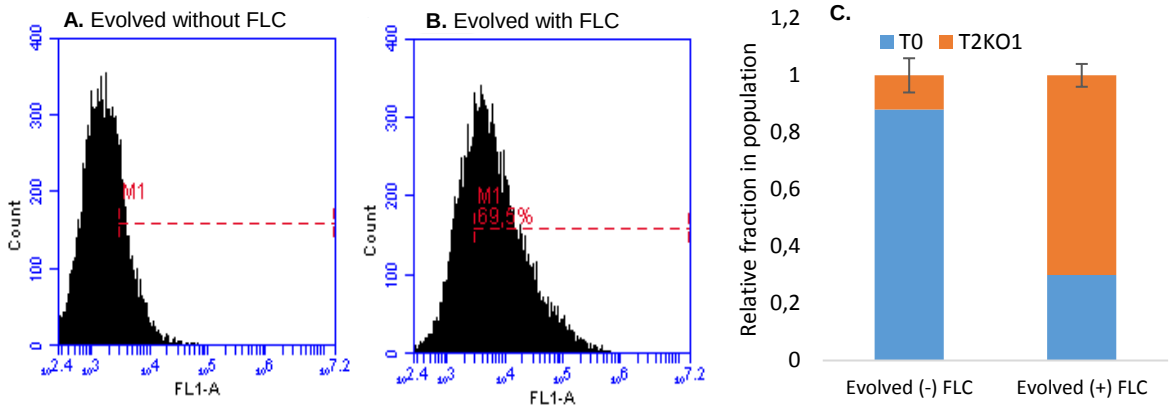

Figure 20. Fluorescence was monitored by flow cytometry at the end of the *in vitro* evolution experiment (400 generations) for competition T0 vs. T2KO1 (A). End of the competition experiment without drug; **(B).** End of the competition experiment with drug ([fluconazole]= 4µg/ml); **(C).** Evolutionary dynamics of experimental populations in the end of the experiment (400 generations) in the absence (no drug) and presence of fluconazole (4µg/ml). The coloured bars represent the relative fractions of each subpopulation – control -strain T0 (blue), mistranslating strain T2KO1 (orange)- as determined by flow cytometry.

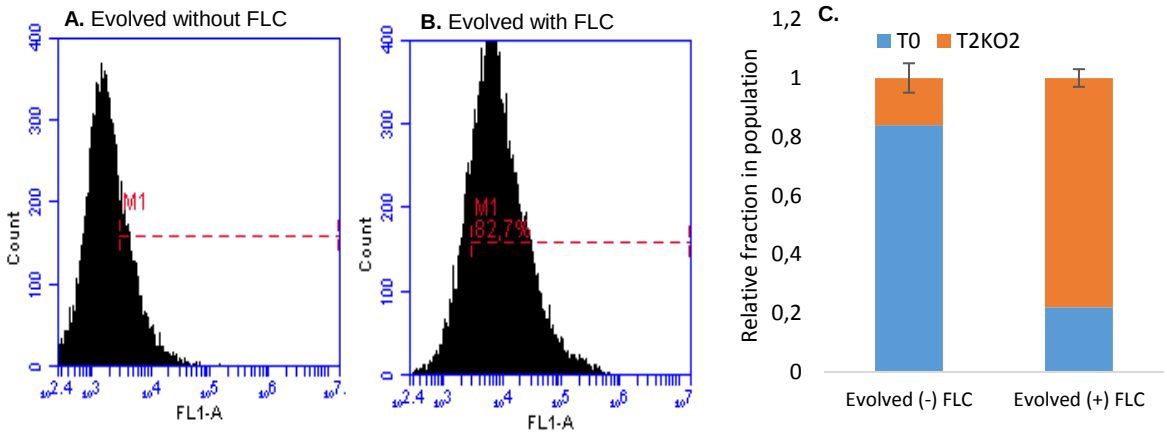

Figure 19. Fluorescence was monitored by flow cytometry at the end of the *in vitro* evolution experiment (400 generations) for competition T0 vs. T2KO2 (A). End of the competition experiment without drug; **(B).** End of the competition experiment with drug ([fluconazole]= 4µg/ml); **(C).** Evolutionary dynamics of experimental populations in the end of the experiment (400 generations) in the absence (no drug) and presence of fluconazole (4µg/ml). The coloured bars represent the relative fractions of each subpopulation – control -strain T0 (blue), mistranslating strain T2KO2 (orange)- as determined by flow cytometry.

3.3 Antifungal susceptibility tests

Susceptibility to fluconazole was monitored at initial and final passages of each competition using two different approaches: the European Committee on Antimicrobial Susceptibility Testing protocol (EUCAST) and the E-test (bioMérieux).

First, we used the initial and final passages from experimental evolution competitions with no drug, fluconazole 0,25 µg/ml and 1 µg/ml to perform the microdilution tests. The values obtained for MICs represent the lowest fluconazole concentration necessary to inhibit the growth of 50% of the population when compared with the growth in the drug free control. The MIC allows fungi to be categorised as 'susceptible', 'intermediate' or 'resistant' to a drug [72]. The breakpoints defined by EUCAST for *C. albicans* using fluconazole are: values of MIC under or equal to 2 µg/ml means that the strain is susceptible and above 4 µg/ml means that is resistant to the drug [73].

The results represented on Table 3 show that in the absence of fluconazole, all the competitions maintained the sensitivity levels both in initial and final passage, with a small variation only in competition T0 vs.T1KO1 in which the MIC was 0,25 µg/ml in the initial passage and increases to 0,5 µg/ml in the final passage.

All competitions that were evolved in the presence of a fluconazole concentration of 0,25 µg/ml showed no alterations of the MIC value between initial and final passages. For competitions in the presence of fluconazole 1 µg/ml, all MICs were the same at the beginning and all of them duplicated the value in the final passage..

Table 3. Competition experimental evolution: MICs were determined in the initial passage and final passage both with and without fluconazole. MIC values were determined using the European Committee on Antimicrobial Susceptibility Testing (EUCAST protocol).

	Without drug (0 µg/ml)		Fluconazole (0,25 µg/ml)		Fluconazole (1 µg/ml)	
	Initial passage MIC_{50} (µg/ml)	Final passage MIC_{50} (µg/ml)	Initial passage MIC_{50} (µg/ml)	Final passage MIC_{50} (µg/ml)	Initial passage MIC_{50} (µg/ml)	Final passage MIC_{50} (µg/ml)
T0 vs T1	0,25	0,25	1,5	1,5	1,5	3
T0 vs T1KO1	0,25	0,5	1,5	1,5	1,5	3
T0 vs T2	0,75	0,75	1,5	1,5	1,5	3
T0 vs T2KO1	0,75	0,75	1,5	1,5	1,5	3
T0 vs T2KO2	0,75	0,75	1,5	1,5	1,5	3

Based on the reference values for susceptibility/resistance, we can see that both on initial and final passages all the MIC results obtained are indicative of susceptible

phenotypes, with exception of the final passage in 1 µg/ml fluconazole where the MIC was 3 µg/ml (indicative of intermediate tolerance phenotype). No resistance phenotypes were detected.

As we looked for the results of microdilution tests, we noted that, on every experiment, there was growth on the wells with concentrations of fluconazole higher than the ones defined as the MIC. As we can see on the table 4, on the highest concentration of fluconazole tested (6 µg/ml) there is still some growth on every competition even though the MIC value was defined as 3 µg/ml. The current clinical recommendations suggest that this growth should be ignored, however, this phenomenon called supra MIC growth (SMG) may indicate tolerance or "trailing growth" [69].

To test if changes in the relative proportion of cells (control *versus* mistranslating) concurred with changes in the tolerance phenotype or "trailing growth" of a population, we determined the minimal inhibitory concentration (MIC) at be beginning and end of the competition evolution using the E-test method. For this test, values of MIC under or equal to 8 µg/ml indicate susceptibility while values above 64 µg/ml are indicative of resistance [74].

Table 4. Results of microdilution test: values of OD_{595} measured for each concentration of fluconazole at the final passage evolved with fluconazole 1 µg/ml. The values at blue represent the MIC. At orange we can see the supra-MIC growth (SMG).

[Fluconazole] (µg/ml)	T0 vs T1	T0 vs T1KO1	T0 vs T2	T0 vs T2KO1	T0 vs T2KO2
0,125	0,8016	0,8916	0,9501	1,0023	1,0681
0,25	0,8912	0,8502	1	1,0127	0,9327
0,5	0,8468	0,8794	0,9350	0,9367	0,8838
0,75	0,9561	0,8272	0,8608	0,7658	0,8332
1,5	0,8271	0,7971	0,9498	0,8770	0,8951
3	0,6942	0,5642	0,5478	0,3599	0,3864
6	0,2459	0,3925	0,2199	0,1306	0,2699

We collected a sample of each competition from the experimental evolution - initial passage and final passages both without drug and with drug (4µg/ml of fluconazole). The E-test results are shown in table 5 and these are slightly different when compared with the values on Table 3. However, we can see that in the tests done to the competitions of the final passage with fluconazole, all MICs are slightly higher than the initial passage with fluconazole, which is similar to the results obtained by the microdilution method.

Table 5. Values of MIC determined by E-test method.

	Without drug (0 µg/ml)		Fluconazole (4µg/ml)	
	Initial passage MIC_{50} (µg/ml)	Final passage MIC_{50} (µg/ml)	Initial passage MIC_{50} (µg/ml)	Final passage MIC_{50} (µg/ml)
T0 vs T1	0,5	1,0	1,5	3,0
T0 vs T1KO1	0,5	1,0	1,0	3,0
T0 vs T2	1,5	1,5	1,5	4,0
T0 vs T2KO1	1,0	2,0	2,0	3,0
T0 vs T2KO2	0,75	0,75	2,0	3,0

Although there is no significative alteration of the global value of susceptibility, and all the MIC values obtained on E-tests are similar to that obtained by the microdilution test and are all included on the reference values of susceptibility (MIC ≤ 8 µg/ml), with the E-test it was possible to see microcolonies appearing on the inhibition halo.

In Figure 21 there is one representative example of competition T0 vs.T2 at the beginning (Figure 21 A.) and at the end of evolution both without drug (figure 21 B.) and with fluconazole 4µg/ml (figure 21 C.) showing the corresponding images of the E-tests.

What we can see is that in the beginning of the experiment (figure 21 A.) there was several well defined microcolonies inside the inhibition ellipse. We picked one of that colonies and analyse on the fluorescence microscope, and we can conclude that inside the halo only grow green cells, which correspond to hypermistranslating cells, in this case T2 cells, which tolerate better the presence of the drug. Outside of the inhibition halo cells grow in a mixture of green (T2) and magenta (T0) cells as we can see in the figure 21 A.

By the end of the experiment (400 generations) without drug (Figure 21 B.), colonies within the inhibition halo disappeared, which correlates well with the fact that without drug, T0 control cells take over the population as seen in the previous section of results. In the case of the evolution with drug (Figure 21 C.), the antifungal treatment selected hypermistranslating cells with drug tolerance phenotypes (as indicated by the persistence of microcolonies within the inhibition ellipse of the E-test in figure 21 C. Once again, these microcolonies are constituted exclusively by green cells, proving that only hypermistranslating cells are able to tolerate the antifungal treatment.

Taken together, these results corroborate the hypothesis that prolonged antifungal therapy favours the appearance of a subpopulation of drug tolerant clones through increased mistranslation.

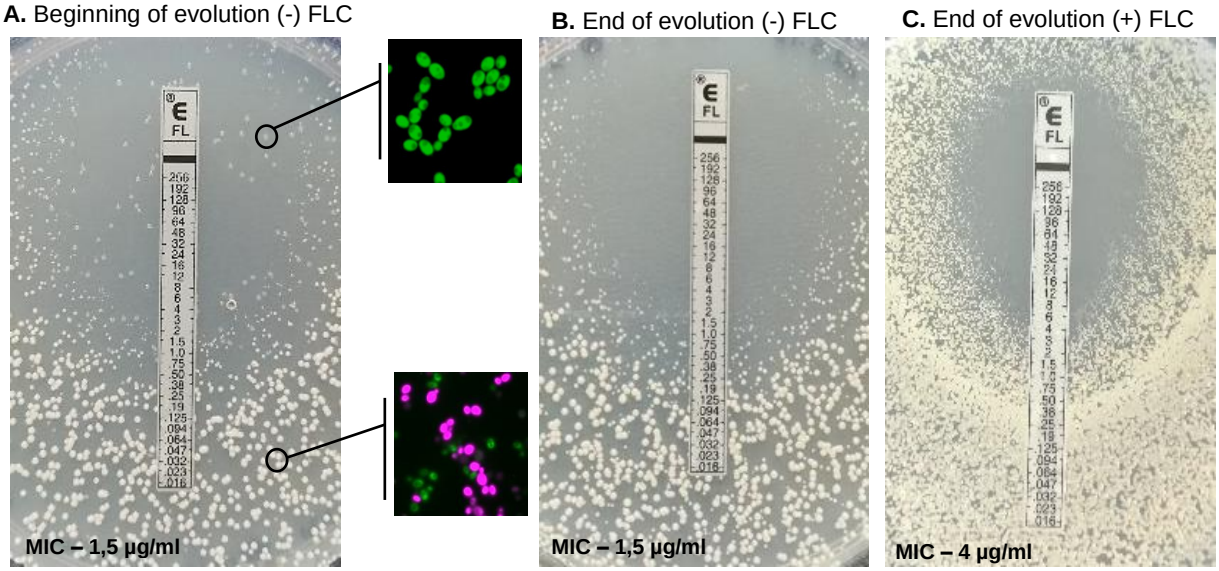

Figure 21. *In vitro* competition (T0-mCherry *versus* T2-GFP) experiments with fluorescently tagged *C. albicans* strains. (A). On the beginning of the experiment there are several microcolonies of green cells on the inside of the inhibition ellipse; **(B).** After 400 generations with no drug, the control population (T0) took over the population and microcolonies disappeared. **(C).** Antifungal therapy selected hypermistranslating cells with drug tolerance phenotypes (as indicated by the maintenance of microcolonies within the inhibition ellipse of the E-test).

4 Discussion

In general, when a drug-treatment fails it is usually attributed to resistance mechanisms. Drug resistance can be defined in distinct ways from clinical and laboratory perspectives. In the clinic context, drug resistance is the persistence of an infection regardless appropriate drug therapy. In the laboratory perspective, drug resistance is quantified by using a clinical MIC assay. The lowest concentration of a drug that inhibits growth by either 50% is defined as the MIC [68]. This means that the growth or survival above inhibitory concentrations (MIC) is rarely quantified, and current clinical recommendations suggest it should be ignored. Although MIC assays provide a measure of how the pathogen will respond to drug treatment, they are not always accurate predictors of the response *in vivo* and do not consider the heterogeneity created by diverse factors on the population.

These groups of cells that overcome drug stress more efficiently than the rest of the population are called “tolerant”. Tolerance, also known as “trailing growth” is sensitive to environmental conditions, including pH, temperature and nutrients [69], [75].

Tolerance and resistance are distinct concepts and also have distinct mechanisms underlying. Mechanisms of resistance have already been described in section 1.5.2. Aneuploidies resulting from genomic instability in *C. albicans* are an example of a common adaptive mechanism by which the pathogen responds to drug-induced stress in order to upregulate genes important for survival [68]. Unlike those, which depend on genes and mutations, mechanisms of tolerance are reversible and result from epigenetic alterations that unleash metabolic alterations, activation of chaperones and signal transduction cascades that sense and respond to stress [75].

For example, alterations on stress pathways like calcineurin signalling pathway are known to control the mechanisms of fluconazole tolerance in *C. albicans.* Sanglard *et al.* has constructed mutants lacking the CNA/CMP1 gene encoding calcineurin subunit A and these mutants turned out to be hypersensitive to fluconazole. Therefore, they established that azole tolerance was mediated by calcineurin activity and its calcium-dependent activation pathway. Inhibition of the chaperone heat shock protein (Hsp90) functions is comparable to a loss of calcineurin activity and therefore has the same effect of calcineurin mutant in drug tolerance [75]–[77].

So far, the majority of studies have been testing new mechanisms of drug resistance in *Candida albicans* as well as new antifungal agents. In the work of Hill *et al*. they evolved experimental populations of *Candida albicans* in the presence of azole and inhibitors of Hsp90 or calcineurin and they found drug target mutations that conferred resistance to those inhibitors in some of the evolved lineages. Their study reveals multiple mechanisms by which resistance to drug combination can evolve and suggest new strategies to combat drug resistance [78]. On the work of Ford *et al.* they saw that aneuploidies and LOH appeared frequently within the drug-resistant isolates and their results suggested that there may be a complex population dynamics during the transition from commensal to pathogen and during the treatment [64].

On the other hand, relatively few evolutionary dynamics studies have been done with pathogenic fungi during *in vitro* evolution of tolerance. On the work of Huang *et al*. they have examined the evolutionary dynamics of *C. albicans* that evolved *in vitro* in the presence or absence of fluconazole using a novel experimental method (visualizing evolution in real-time) that facilitates the systematic isolation of adaptive mutants that arise in the population. They found an increase in the frequency of adaptive events in the presence of fluconazole compared to the no-drug controls [79].

Also, studies made in Mycobacteria have found a relation between mistranslation and resistance to antibiotics. On the work of Javid *et al.* they found that mycobacteria substitute glutamate for glutamine and aspartate for aspargine at high rates under specific growth conditions. The increasing of mistranslation resulted in “phenotypic resistance” to rifampicin, whereas decreasing mistranslation produces increased susceptibility to the antibiotic. On their work they made the distinction between phenotypic drug resistance or tolerance and genetic resistance highlighting the fact that resistant organisms have a normal growth in the presence of drug while resistant cells have a reduction of growth. Moreover, they highlighted that even if drug-tolerant bacteria do grow in antibiotic, not all of their progeny will survive antibiotic action, which proves that this alterations are not due to genetic alterations and can represent a form of environmental adaptation [80].

In our study, we used different strains of *C. albicans* in competition pairs to help us understand if hypermistranslating strains are favoured in the presence of drug when compared with a susceptible control strain (T0). Results showed that in the end of the evolution in the presence of fluconazole, in general, hypermistranslating strains (T2, T2KO1 and T2KO2) are the dominant fraction of the population. These alterations in the relative proportion of a population (control vs mistranslating) concurred with the appearance of

tolerance phenotypes (appearance of trailing growth). Although the mechanism behind such phenotype was not unveiled, we can speculate that it may involve the calcineurin pathway. In the work of Bezerra *et al*., the genome sequencing data of mistranslating strains did not show accumulation of mutations in ergosterol pathway genes, suggesting that mistranslation-induced drug tolerance is independent of mutations in known drug target genes [27]. However, the partial loss of heterozygosity (LOH) on chromosome V of the high mistranslating strains, which affected the regulatory subunit of calcineurin (CNB1) and the calcium channel (MID1), supports the hypothesis that such tolerance may be mediated through the calcineurin pathway [27].

Other hypotheses may involve intracellular drug accumulation and/or slow growth. In the work of Rosenberg *et al.*, they quantified antifungal tolerance in *Candida albicans* isolates as the fraction of growth (FoG) above the MIC and verified that the increase of tolerance is related with the decrease of intracellular drug accumulation [69]. In the case of our mistranslating strains, that also may be the case. Weil *et al.* explored the mechanisms that lead to a rapid acquisition of drug resistance under mistranslation. They performed genome sequencing, array-based comparative genome analysis, and gene expression profiling of our mistranslating strain T1 and verified that during the course of evolution in fluconazole, the range of mutational and gene deregulation differences was higher in the hypermistranslating strain T1 when compared to control T0. Of particular interest was the fact that T1 overexpressed the ABC superfamily transporter genes CDR1 and CDR2 that are responsible for efflux of the drug [71].

Tolerance phenotypes of non-growing or slow-growing bacteria that can survive the action of antibiotics was described by Reisman *et al.* [81]. These mechanisms that help to survive drug exposure are also seen in our strains. Hypermistranslating strains (T1, T1KO1, T2, T2KO1 and T2KO2) have a slow growth rate when compared with the control strain (T0) which could be related with the appearance of subpopulations with tolerance phenotypes seen in our work.

Subpopulations of *Candida albicans* with tolerance phenotypes have also been described in the work of Rosenberg *et al.*. They showed that "tolerance" can be detected as slow growth of subpopulations within the zone of inhibition. They also made the association between highly tolerant isolates and persistent candidemia, suggesting that knowing the tolerance level of an infecting isolate may have important clinical implications and may help choose the best options for treatment [69].

Resistance to azoles, including fluconazole, the most commonly administered antifungal against *Candida* species, is an increasing problem. In this experimental study we suggest that mistranslation may comprise a new way of generating tolerance. The growth of tolerant subpopulations of *C. albicans* should not be ignored when the MICs are measured because these subpopulations of "persisters" may be the reason why sometimes antifungal therapies fail. Tolerant cells continue to divide in the presence of antifungals contributing to the persistence and/or recurrence of fungal infections [69].

One of the strategies that can be used to fight the tolerance is the use of adjuvant drugs in combination with fluconazole. There are evidences resulting from the work of Rosenberg *et al.* that show that adjuvant combinations with fluconazole clear tolerance (trailing growth) and do not affect the resistance. Some adjuvants like geldanamycin and radicicol inhibit the heat shock protein 90 (Hsp90) activity; cyclosporine A and FK506 inhibits calcineurin and staurosporine inhibits protein kinase C1 (Pkc1) activity, among others. And all of them significantly reduced FoG, which means that they have a profound effect on cell viability at supra-MIC fluconazole concentrations and implying that stress pathways make essential contributions to tolerance [69]. In the future it will be interesting to test the use of adjuvant drugs in combination with fluconazole on our strains.

Finally, some studies made in bacteria suggested that antibiotic tolerance may benefit the evolution of resistance. Levin-Reisman *et al*. used *Escherichia coli* cells repeatedly treated with antibiotic and they found that resistance developed faster if the starting strain carried a tolerance phenotype. This association between tolerance and resistance highlights the need to prevent the evolution of tolerance by the development of compounds that can eliminate them. The discovery of a link between mistranslation, tolerance and resistance may offer a new strategy for delaying the emergence of resistance [81].

5 Conclusions and future perspectives

Our results suggest that mistranslation may play a fundamental role in fungal drug tolerance. During our experimental evolution experiment, we showed that a population segregates into “hypermistranslator” and relatively “wild-type” cell types upon challenge with fluconazole. The appearance of these “hypermistranslator” subpopulations of cells coincided with phenotypes of tolerance, characterized by the slow growth of cells that overcome drug stress more efficiently than the rest of the population. By other words, prolonged antifungal therapy selects drug tolerant cells through mistranslation.

The genomes of the hypermistranslating strains experimentally evolved *in vitro* should be sequenced in order to understand the genetic alterations underlying this enhanced tolerance to antifungal therapy, however, since most of the times, the development of mechanisms of tolerance are not due to genetic alterations, it may also be useful to analyse their transcriptome. Future works may also include tests with adjuvant drugs in combination with fluconazole to see how they alter the tolerance of our strains and measurements of the intracellular drug accumulation in our evolved strains.

In the future it will be interesting to understand how these alterations responsible for the appearance of tolerant subpopulations may be associated with the development of phenotypes of resistance.

Bibliography

[1] K. E. van Holde and J. Zlatanova, “The Central Dogma,” *Evol. Mol. Biol.*, pp. 87–94, 2018.

[2] M. Chekulaeva and M. Landthaler, “Eyes on Translation,” *Mol. Cell*, vol. 63, no. 6, pp. 918–925, 2016.

[3] N. M. Reynolds, B. A. Lazazzera, and M. Ibba, “Cellular mechanisms that control mistranslation,” *Nat. Rev. Microbiol.*, vol. 8, no. 12, pp. 849–856, 2010.

[4] J. E. Richards and R. S. Hawley, The Central Dogma of Molecular Biology: How Cells Orchestrate the Use of Genetic Information. 2011.

[5] A. G. Hinnebusch, “Molecular Mechanism of Scanning and Start Codon Selection in Eukaryotes,” vol. 75, no. 3, pp. 434–467, 2011.

[6] L. D. Kapp and J. R. Lorsch, “The molecular mechanics of eukaryotic translation,” pp. 657–704, 2004.

[7] A. Saint-Léger and L. Ribas de Pouplana, “The importance of codon e anticodon interactions in translation elongation,” vol. 114, pp. 72–79, 2015.

[8] F. H. C. Crick, “The Origin of the Genetic Code,” *J. Mol. Biol.*, pp. 367–379, 1968.

[9] P. J. Keeling, “Genomics : Evolution of the Genetic Code,” *Curr. Biol.*, vol. 26, no. 18, pp. R851–R853, 2016.

[10] E. V. Koonin and A. S. Novozhilov, “Origin and Evolution of the Universal Genetic Code,” *Rev Can Biol Exp*, vol. 41, no. 3, pp. 209–216, 2017.

[11] A. C. Gomes, I. Miranda, R. Rocha, M. C. Santos, D. D. Mateus, G. R. Moura, L. Carreto, and M. A. S. Santos, “A genetic code alteration generates a proteome of high diversity in the human pathogen Candida albicans,” *PLoS One*, vol. 2, no. 10, 2007.

[12] I. Miranda, R. Rocha, M. C. Santos, D. D. Mateus, G. R. Moura, L. Carreto, and M. A. S. Santos, “A Genetic Code Alteration Is a Phenotype Diversity Generator in the Human Pathogen Candida albicans,” no. 10, 2007.

[13] S. Sengupta, X. Yang, and P. G. Higgs, “The Mechanisms of Codon Reassignments in Mitochondrial Genetic Codes,” pp. 662–688, 2007.

[14] J. S. Foltan, “tRNA genes and the genetic code,” *J. Theor. Biol.*, vol. 253, no. 3, pp. 469–482, 2008.

[15] S. D. Banik and N. Nandi, “Influence of the conserved active site residues of histidyl tRNA synthetase on the mechanism of aminoacylation reaction,” *Biophys. Chem.*, vol. 158, no. 1, pp. 61–72, 2011.

[16] P. J. Beuning and K. Musier-Forsyth, “Transfer RNA recognition by aminoacyl-tRNA

synthetases," *Biopolymers*, vol. 52, no. 1, pp. 1–28, 1999.

[17] M. Schwartz and T. Pan, "Function and origin of mistranslation in distinct cellular contexts," vol. 52, no. 2, pp. 205–219, 2018.

[18] Y. L. J. Pang and S. A. Martinis, "A Paradigm Shift for the Amino Acid Editing Mechanism of Human Cytoplasmic Leucyl-tRNA Synthetase," *NIH Public Access*, vol. 48, no. 38, pp. 8958–8964, 2010.

[19] H. Jakubowski, "Quality control in tRNA charging," *Wiley Interdiscip. Rev. RNA*, vol. 3, no. 3, pp. 295–310, 2012.

[20] S. Pande, A. Vimaladithan, H. Zhao, and P. J. Farabaugh, "Pulling the ribosome out of frame by +1 at a programmed frameshift site by cognate binding of aminoacyl-tRNA.," *Mol. Cell. Biol.*, vol. 15, no. 1, pp. 298–304, 1995.

[21] R. De Pouplana, M. A. S. Santos, J. Zhu, and P. J. Farabaugh, "Protein mistranslation : friend or foe ?," vol. 39, no. 8, pp. 355–362, 2014.

[22] J. W. Lee, K. Beebe, L. A. Nangle, J. Jang, C. M. Longo-guess, S. A. Cook, M. T. Davisson, J. P. Sundberg, P. Schimmel, and S. L. Ackerman, "Editing-defective tRNA synthetase causes protein misfolding and neurodegeneration," no. October, 2006.

[23] L. A. Nangle, D. Cre, V. Do, and P. Schimmel, "Genetic Code Ambiguity. Cell viability related to severity of editing defects in mutant tRNA synthetases," vol. 277, no. 48, pp. 45729–45733, 2002.

[24] J. M. Bacher, V. Crécy-Lagard, and P. R. Schimmel, "Inhibited cell growth and protein functional changes from an editing-defective tRNA synthetase," no. 12, 2005.

[25] M. F. Tuite and M. A. S. Santos, "Codon reassignment in Candida species: An evolutionary conundrum," *Biochimie*, vol. 78, no. 11–12, pp. 993–999, 1996.

[26] M. A. S. Santos, C. Cheesman, V. Costa, P. Moradas-Ferreira, and M. F. Tuite, "Selective advantages created by codon ambiguity allowed for the evolution of an alternative genetic code in Candida spp.," *Mol. Microbiol.*, vol. 31, no. 3, pp. 937–947, 1999.

[27] A. R. Bezerra, J. Simoes, W. Lee, J. Rung, T. Weil, I. G. Gut, M. Gut, M. Bayes, L. Rizzetto, D. Cavalieri, G. Giovannini, S. Bozza, L. Romani, M. Kapushesky, G. R. Moura, and M. A. S. Santos, "Reversion of a fungal genetic code alteration links proteome instability with genomic and phenotypic diversification," *Proc. Natl. Acad. Sci.*, vol. 110, no. 27, pp. 11079–11084, 2013.

[28] I. Miranda, R. Silva, and M. A. S. Santos, "Evolution of the genetic code in yeasts," *Yeast*, vol. 23, no. 3, pp. 203–213, 2006.

[29] I. Miranda, A. Silva-Dias, R. Rocha, R. Teixeira-Santos, C. Coelho, T. Gonçalves, and M. A.

S. Santos, “Candida albicans CUG Mistranslation Is a Mechanism To Create Cell Surface Variation,” vol. 4, no. 4, pp. 1–9, 2013.

[30] M. Dadar, R. Tiwari, K. Karthik, S. Chakraborty, Y. Shahali, and K. Dhama, “Candida albicans - Biology, molecular characterization, pathogenicity, and advances in diagnosis and control – An update,” *Microb. Pathog.*, vol. 117, no. February, pp. 128–138, 2018.

[31] B. A. McManus and D. C. Coleman, “Molecular epidemiology, phylogeny and evolution of Candida albicans,” *Infect. Genet. Evol.*, vol. 21, pp. 166–178, 2014.

[32] Z. Sárkány, A. Silva, P. J. B. Pereira, and S. Macedo-Ribeiro, “Ser or Leu: structural snapshots of mistranslation in Candida albicans,” *Front. Mol. Biosci.*, vol. 1, no. December, pp. 1–14, 2014.

[33] L. S. Rai, R. Singha, P. Brahma, and K. Sanyal, “Epigenetic determinants of phenotypic plasticity in Candida albicans,” *Fungal Biol. Rev.*, vol. 32, no. 1, pp. 10–19, 2018.

[34] S. M. Noble, B. A. Gianetti, and J. N. Witchley, “Candida albicans cell type switches and functional plasticity in the mammalian host,” vol. 15, no. 2, pp. 96–108, 2017.

[35] I. D. Jacobsen, D. Wilson, B. Wächtler, S. Brunke, J. R. Naglik, and B. Hube, “Candida albicans dimorphism as a therapeutic target,” *Expert Rev. Anti. Infect. Ther.*, vol. 10, no. 1, pp. 85–93, 2012.

[36] J. Kim and P. Sudbery, “Candida albicans, a major human fungal pathogen,” *J. Microbiol.*, vol. 49, no. 2, pp. 171–177, 2011.

[37] F. L. Mayer, D. Wilson, and B. Hube, “Candida albicans pathogenicity mechanisms,” *Virulence*, vol. 4, no. 2, pp. 119–28, 2013.

[38] A. Kumar and A. Jha, “Chapter 4 - Drug Resistance in Candida,” *Anticandidal Agents*, pp. 41–47, 2017.

[39] C. M. Hull, R. M. Raisner, and A. D. Johnson, “Evidence for mating of the ‘asexual’ yeast Candida albicans in a mammalian host.,” *Science*, vol. 289, no. 5477, pp. 307–10, 2000.

[40] R. J. Bennett, “The parasexual lifestyle of Candida albicans,” *Curr. Opin. Microbiol.*, vol. 28, pp. 10–17, 2015.

[41] M. B. Lohse and A. D. Johnson, “White-opaque switching in Candida albicans,” *Curr. Opin. Microbiol.*, vol. 12, no. 6, pp. 650–654, 2009.

[42] M. Polke, B. Hube, and I. D. Jacobsen, *Candida survival strategies*, vol. 91. Elsevier Ltd, 2015.

[43] M. A. Hickman, G. Zeng, A. Forche, M. P. Hirakawa, D. Abbey, B. D. Harrison, Y. M. Wang, C. H. Su, R. J. Bennett, Y. Wang, and J. Berman, “The ‘obligate diploid’ Candida albicans forms mating-competent haploids,” *Nature*, vol. 494, no. 7435, pp. 55–59, 2013.

[44] G. D. Brown, D. W. Denning, N. A. R. Gow, S. M. Levitz, M. G. Netea, and T. C. White, “Hidden killers: Human fungal infections,” *Sci. Transl. Med.*, vol. 4, no. 165, 2012.

[45] A. Nantel, “The long hard road to a completed Candida albicans genome,” *Fungal Genet. Biol.*, vol. 43, no. 5, pp. 311–315, 2006.

[46] R. J. Bennett and A. D. Johnson, “Mating in Candida albicans and the Search for a Sexual Cycle,” *Annu. Rev. Microbiol.*, vol. 59, no. 1, pp. 233–255, 2005.

[47] R. Garcia-Rubio, M. C. Monteiro, and E. Mellado, *Azole Antifungal Drugs: Mode of Action and Resistance*. Elsevier Ltd., 2018.

[48] A. da Silva Dantas, K. K. Lee, I. Raziunaite, K. Schaefer, J. Wagener, B. Yadav, and N. A. Gow, “Cell biology of Candida albicans–host interactions,” *Curr. Opin. Microbiol.*, vol. 34, pp. 111–118, 2016.

[49] S. Y. Ting, O. A. Ishola, M. A. Ahmed, Y. M. Tabana, S. Dahham, M. T. Agha, S. F. Musa, R. Muhammed, L. T. L. Than, and D. Sandai, “Metabolic adaptation via regulated enzyme degradation in the pathogenic yeast Candida albicans,” *J. Mycol. Med.*, vol. 27, no. 1, pp. 98–108, 2017.

[50] B. Hube, “Fungal adaptation to the host environment Editorial overview,” pp. 347–349, 2009.

[51] M. Gulati and C. J. Nobile, “Candida albicans biofilms: development, regulation, and molecular mechanisms,” *Microbes Infect.*, vol. 18, no. 5, pp. 310–321, 2016.

[52] J. Bonhomme and C. d’Enfert, “Candida albicans biofilms: Building a heterogeneous, drug-tolerant environment,” *Curr. Opin. Microbiol.*, vol. 16, no. 4, pp. 398–403, 2013.

[53] D. L. Moyes, J. P. Richardson, J. R. Naglik, D. L. Moyes, J. P. Richardson, J. R. N. Candida, F. Group, L. L. C. D. L. Moyes, J. P. Richardson, J. R. Naglik, D. L. Moyes, J. P. Richardson, and J. R. Naglik, “Candida albicans- epithelial interactions and pathogenicity mechanisms: scratching the surface,” vol. 5594, no. October 2016, pp. 338–346, 2015.

[54] N. A. Gow and B. Hube, “Importance of the Candida albicans cell wall during commensalism and infection,” *Curr. Opin. Microbiol.*, vol. 15, no. 4, pp. 406–412, 2012.

[55] D. Poulain, “Candida albicans, plasticity and pathogenesis,” *Crit. Rev. Microbiol.*, vol. 41, no. 2, pp. 208–217, 2015.

[56] R. I. Benhamou, M. Bibi, K. B. Steinbuch, H. Engel, M. Levin, Y. Roichman, J. Berman, and M. Fridman, “Real-Time Imaging of the Azole-Class of Antifungal Drugs in Live Candida Cells,” 2017.

[57] G. C. d. O. Santos, C. C. Vasconcelos, A. J. O. Lopes, M. do S. d. S. Cartágenes, A. K. D. B. Filho, F. R. F. do Nascimento, R. M. Ramos, E. R. R. B. Pires, M. S. de Andrade, F. M. G. Rocha, and C. de A. Monteiro, “Candida infections and therapeutic strategies: Mechanisms

of action for traditional and alternative agents," *Front. Microbiol.*, vol. 9, no. JUL, pp. 1–23, 2018.

[58] A. Kumar and A. Jha, "Antifungals Used Against Candidiasis," *Anticandidal Agents*, pp. 11–39, 2017.

[59] N. M. Revie, K. R. Iyer, N. Robbins, and L. E. Cowen, "Antifungal drug resistance: evolution, mechanisms and impact," *Curr. Opin. Microbiol.*, vol. 45, no. Figure 2, pp. 70–76, 2018.

[60] J. Pemán, E. Cantón, and A. Espinel-Ingroff, "Antifungal drug resistance mechanisms," *Expert Rev. Anti. Infect. Ther.*, vol. 7, no. 4, pp. 453–460, 2009.

[61] D. S. Perlin, R. Rautemaa-Richardson, and A. Alastruey-Izquierdo, "The global problem of antifungal resistance: prevalence, mechanisms, and management," *Lancet Infect. Dis.*, vol. 17, no. 12, pp. e383–e392, 2017.

[62] K. Y. Lin, P. Y. Chen, Y. C. Chuang, J. T. Wang, H. Y. Sun, W. H. Sheng, Y. C. Chen, and S. C. Chang, "Effectiveness of echinocandins versus fluconazole for treatment of persistent candidemia: A time-dependent analysis," *J. Infect.*, vol. 77, no. 3, pp. 242–248, 2018.

[63] J. E. Nett and D. R. Andes, "Antifungal Agents: Spectrum of Activity, Pharmacology, and Clinical Indications," *Infect. Dis. Clin. North Am.*, vol. 30, no. 1, pp. 51–83, 2016.

[64] C. B. Ford, J. M. Funt, D. Abbey, L. Issi, C. Guiducci, D. A. Martinez, T. Delorey, B. Y. Li, T. White, C. Cuomo, R. P. Rao, J. Berman, D. A. Thompson, and A. Regev, "The evolution of drug resistance in clinical isolates of candida albicans," *Elife*, vol. 2015, no. 4, pp. 1–27, 2015.

[65] T. Noël, "The cellular and molecular defense mechanisms of the Candida yeasts against azole antifungal drugs," *J. Mycol. Med.*, vol. 22, no. 2, pp. 173–178, 2012.

[66] B. Wang, L. H. Huang, J. X. Zhao, M. Wei, H. Fang, D. Y. Wang, H. F. Wang, J. G. Yin, and M. Xiang, "ERG11 mutations associated with azole resistance in Candida albicans isolates from vulvovaginal candidosis patients," *Asian Pac. J. Trop. Biomed.*, vol. 5, no. 11, pp. 909–914, 2015.

[67] J. L. Xie, E. J. Polvi, T. Shekhar-guturja, and L. E. Cowen, "Elucidating drug resistance in human fungal pathogens," vol. 9, pp. 523–542, 2014.

[68] R. S. Shapiro, N. Robbins, and L. E. Cowen, "Regulatory Circuitry Governing Fungal Development, Drug Resistance, and Disease," *Microbiol. Mol. Biol. Rev.*, vol. 75, no. 2, pp. 213–267, 2011.

[69] A. Rosenberg, I. V. Ene, M. Bibi, S. Zakin, E. S. Segal, N. Ziv, A. M. Dahan, A. L. Colombo, R. J. Bennett, and J. Berman, "Antifungal tolerance is a subpopulation effect distinct from resistance and is associated with persistent candidemia," *Nat. Commun.*, vol. 9, no. 1, 2018.

[70] M. D. LaFleur, "Candida albicans Biofilms , Heterogeneity and Antifungal Drug Tolerance,"

pp. 21–28, 2011.

[71] T. Weil, R. Santamaria, W. Lee, J. Rung, N. Tocci, D. Abbey, A. R. Bezerra, L. Carreto, G. R. Moura, M. Bayés, I. G. Gut, A. Csikasz-Nagy, D. Cavalieri, J. Berman, and M. A. S. Santos, “Adaptive Mistranslation Accelerates the Evolution of Fluconazole Resistance and Induces Major Genomic and Gene Expression Alterations in Candida albicans,” vol. 2, no. 4, pp. 1–14, 2017.

[72] EUCAST, “Method for the determination of broth dilution MICs of antifungal agents for fermentative yeasts,” *Clin Microbiol Infect*, vol. 14, pp. 398–405, 2008.

[73] EUCAST, “Breakpoint tables for interpretation of MICs, Version 8.1, valid from 2017-03-01,” pp. 1–5, 2017.

[74] AB BIODISK, “Etest for Antifungal Susceptibility Testing,” *Ab Biodisk*, 2000.

[75] E. Delarze and D. Sanglard, “Defining the frontiers between antifungal resistance, tolerance and the concept of persistence,” vol. 23, no. 3, pp. 12–19, 2015.

[76] P. Uppuluri, J. Nett, J. Heitman, and D. Andes, “Synergistic Effect of Calcineurin Inhibitors and Fluconazole against,” vol. 52, no. 3, pp. 1127–1132, 2008.

[77] D. Sanglard, F. Ischer, O. Marchetti, J. Entenza, and J. Bille, “Calcineurin A of Candida albicans: Involvement in antifungal tolerance, cell morphogenesis and virulence,” *Mol. Microbiol.*, vol. 48, no. 4, pp. 959–976, 2003.

[78] J. A. Hill, R. Ammar, D. Torti, C. Nislow, and L. E. Cowen, “Genetic and Genomic Architecture of the Evolution of Resistance to Antifungal Drug Combinations,” *PLoS Genet.*, vol. 9, no. 4, 2013.

[79] M. Huang, M. McClellan, J. Berman, and K. C. Kao, “Evolutionary dynamics of candida albicans during in vitro evolution,” *Eukaryot. Cell*, vol. 10, no. 11, pp. 1413–1421, 2011.

[80] B. Javid, F. Sorrentino, M. Toosky, W. Zheng, J. T. Pinkham, N. Jain, and M. Pan, “Mycobacterial mistranslation is necessary and sufficient for rifampicin phenotypic resistance,” 2013.

[81] I. Levin-Reisman, I. Ronin, O. Gefen, I. Braniss, N. Shoresh, and N. Q. Balaban, “Antibiotic tolerance facilitates the evolution of resistance,” *Science (80-.).*, vol. 355, no. 6327, pp. 826–830, 2017.

Annexes

Table 6. Images of all the E-test performed. The test was performed for each competition on the initial and final passages of the experimental evolution and both with no drug and with drug (4µg/ml of fluconazole).

	Without drug		With drug (4µg/ml of fluconazole)	
	Initial passage	Final passage	Initial passage	Final passage
T0 vs T1				
T0 vs T1KO1				
T0 vs T2				
T0 vs T2KO1				
T0 vs T2KO2