Universidade de Aveiro
Ano 2019

Departamento de Ciências Médicas

Matilde de Almeida Figueiredo

The role of mistranslation in the acquisition of antifungal resistance

O papel dos erros de tradução na aquisição de resistência a antifúngicos

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The role of mistranslation in the acquisition of antifungal resistance

O papel dos erros de tradução na aquisição de resistência a antifúngicos

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 through UID/BIM/04501/2019, projects POCI-01-0145-FEDER-007628, POCI-01-0145-FEDER-022184 and grant PTDC/IMI-MIC/5350/2014.

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Doutor João Manuel Salvador Simões
Investigador Coordenador da Unidade Genómica do Biocant

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

Agradecimentos

Agradeço ao Professor Doutor Manuel Santos por ter permitido que este trabalho fosse possível. À minha orientadora Dr. Ana Rita Bezerra, por todos os conhecimentos que me transmitiu, pela sua pronta ajuda, e pela dedicação e disponibilidade que sempre teve comigo.

Ás minhas colegas de laboratório, pela sua simpatia e paciência, por me terem sempre ajudado em qualquer dúvida no trabalho experimental e pelos conhecimentos que me transmitiram.

A todas as minhas amigas do Mestrado, por todo o companheirismo ao longo destes dois anos.

Um especial agradecimento aos meus pais, por todo o apoio e opurtunidades que sempre me deram, por toda a preocupação, pelas palavras de apoio e incentivo.

Agradeço ainda ao meu namorado pelo seu carinho e amor, pela sua preocupação e apoio nesta caminhada.

palavras-chave *Candida albicans*, evolução experimental, erros de tradução, resistência a drogas

resumo A tradução do mRNA pelo ribossoma é um processo biológico de elevada fidelidade cujo erro basal é da ordem de 10^{-3} a 10^{-4} em células eucarióticas. Este baixo nível de erro generalizou a ideia de que os erros de tradução não têm relevância biológica. Contudo, estudos recentes mostram que tais erros são regulados e são relevantes para a adaptação, em particular em situações de stress. *Candida albicans*, o fungo patogénico mais prevalente nos humanos, traduz naturalmente o mRNA com elevado nível de erro de modo dinâmico. A exposição de *C. albicans* a diferentes condições de crescimento aumenta o nível de erro de tradução o que, por sua vez, induz a diversificação do seu genoma e o aparecimento de fenótipos de tolerância a antifúngicos. Neste estudo, colocámos a hipótese de que os erros de tradução aceleram a aquisição de resistência ao fluconazol através de alterações genómicas. Para testar esta hipótese, evoluímos estirpes com elevado erro de tradução e estirpes controlo na ausência e presença de fluconazol e comparámos os seus perfis de resistência durante a evolução. Os resultados mostram que os erros de tradução proporcionam um aumento da frequência de aquisição de resistência ao fluconazol. A avaliação da ploidia e sequenciação dos genomas revelaram que durante a evolução com droga, a diversificação do genoma foi maior nas estirpes com elevado erro de tradução. Especificamente, mutações nos genes de efluxo, alvos da droga e biossíntese de ergosterol parecem acelerar a aquisição de resistência ao fluconazol nestas estirpes. O presente trabalho revela o papel central dos erros de tradução como mecanismo de adaptação dos fungos patogénicos aos antimicóticos.

Keywords *Candida albicans*, experimental evolution, mistranslation, drug resistance

Abstract

Translation of mRNA by the ribosome is a high-fidelity biological process whose error rates range from 10^{-3} to 10^{-4} in eukaryotic cells. Such low error rate generalized the idea that mistranslation is a nuisance to the cell without biological relevance. However, recent studies show that translational errors are regulated and play important roles in adaptation to stress situations. *Candida albicans*, the most prevalent human fungal pathogen, mistranslates naturally at high level in a dynamic way. Exposure of *C. albicans* to different growth conditions increases mistranslation levels which results in genomic diversification and increased tolerance to antifungals. In this study, we hypothesized that mistranslation accelerates the acquisition of resistance to fluconazole through genomic alterations. To test this hypothesis, we evolved hypermistranslating and wild-type strains in the absence and presence of fluconazole and compared their resistance trajectories during evolution. Results show that mistranslation increases the frequency of acquisition of fluconazole resistance. Ploidy assessment and genome sequencing revealed that during the course of evolution in fluconazole, the range of genomic diversification was broader in the hypermistranslating strains. Specifically, mutations in efflux, drug target and ergosterol biosynthesis genes seem to speed up the acquisition of antifungal drug resistance in hypermistranslating strains. The present work reveals the pivotal role of mistranslation as a mechanism of adaptation of pathogens to antimicrobials.

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1 Introduction

1.1. Protein synthesis

In 1960s, the central dogma had been accepted as the pathway in which information flows from genes to proteins. It was accepted that genes encode proteins, that genes are made of DNA molecules and that the mRNA acts as an intermediary that carries the information from DNA to the ribosome, where proteins are synthesised [1].

The information contained in genes directs the synthesis of proteins, which are the main constituents of cells, responsible for proper cell structure and function. Protein synthesis is the mechanism of protein production from the information present in the DNA. It involves two main steps: transcription, in which an RNA molecule (mRNA) is produced from the DNA, taking place in the nucleus, and translation in which that mRNA is used to produce proteins in the cytoplasm (Figure 1) [1].

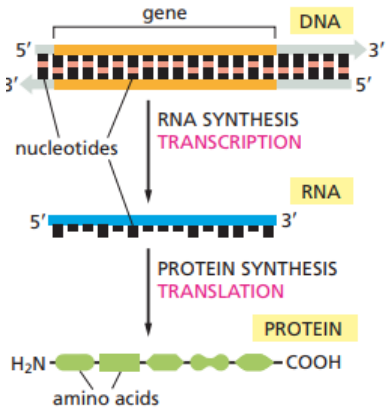

Figure 1- The flow of genetic information. Genetic information flows from DNA to RNA (transcription) and from RNA to protein (translation). Adapted from [1].

The genetic code determines the rule of translation of nucleotide sequences, the mRNA, into amino acids that form proteins. According to the genetic code there are four different ribonucleotide bases in the mRNA A (adenin), C (cytocin), U (uracil), G (guanine), that are arranged into triplets/codons (A pairs with U and C pairs with G), forming in total 64 codons. Of these, 61 encode the 20 standard amino acids, and 3 (UAA, UAG, UGA) are stop codons [1]. Thus, the genetic code is said to be degenerate as the same amino acid can be specified

by more than one codon triplet that differ in the third base, designated the “wobble position”. Such codons, that encode for the same amino acid are said to be synonymous. However, there are two exceptions: Met (methionine) and Trp (tryptofan), which are only specified by one codon each, AUG and UGG respectively. The different synonymous codons aren’t used in the same frequencies, some are more used than others, creating a codon usage bias, which in turn influences gene expression and cellular function [7][8][9].

Besides mRNA, there are other types of RNA molecules involved in protein synthesis. The ribosomal RNAs (rRNAs) are a part of the structure of the ribosomes and they translate mRNAs into protein. Transfer RNAs (tRNAs) act as adaptor molecules that implement the genetic code as they recognize and bind to an mRNA codon and participate in the selection of the corresponding amino acid as they hold them in place on a ribosome for their incorporation into proteins [1]. The tRNA molecules have a region, the anticodon, that binds to the complementary codon in the mRNA through base-pairing, being therefore crucial for protein synthesis. The amino-acid corresponding to the codon-anticodon pair is attached to a single-stranded region in the 3’ end of the tRNA by a specific enzyme, the aminoacyl tRNA-synthetase (aaRS). This enzyme recognizes determinant regions of the tRNA molecule. Thus, each aaRS has a specific amino acid identity and is responsible for charging a single type of amino acid to the cognate tRNAs. In most organisms there is one aminoacyl-tRNA-synthetase for each amino-acid [1][10][2].

Translation from mRNA to protein takes place in the ribosomes, which act as machines that move along the mRNA, capture the complementary tRNAs, and add the corresponding amino acids, producing a polypeptide chain. Ribosomes have two different subunits involved in tRNA recognition and elongation of the polypeptide chain, the smaller 40S subunit (or 30S in prokaryotes) and the larger 60S subunit (or 50S in prokaryotes). The smaller subunit contains the decoding site where the mRNA codons interact with tRNA anticodons; whereas the larger subunit contains the active site where peptide bonds covalently link the amino acids together into a polypeptide chain. During translation initiation these two subunits come together on an mRNA molecule, forming the 80S (or 70S in eukaryotes) ribosome. The ribosome structure has a biding site for the mRNA molecule and three binding sites for the tRNA, the acyl site (A), the peptidyl site (P) and the exit site (E) [1]. The translation process requires other proteins extrinsic to the ribosome, which are known as translation factors [11].

The translation mechanism involves four steps: translation initiation, elongation, termination and recycling (Figure 2). Translation initiation requires the recognition of a specific start

sequence in the mRNA, the AUG codon, that sets the reading frame of the translated message. After the recognition of this codon, the small ribosome subunit positions the initiator tRNA charged with the amino acid methionine, over the mRNA's start codon- AUG- in the P site. In the elongation phase, the correct amino acids are added to the growing polypeptide chain, until a stop codon in the mRNA appears, which ends the growing phase. Then, in the termination step, the polypeptide chain formed is released from the ribosome; and lastly, in the recycling phase, the ribosome is released from the tRNA and mRNA [12][7][1][11].

The process of adding an amino acid to the growing peptide chain, begins with positioning the correct charged tRNA in the A-site by base-pairing with the complementary codon on the mRNA. The amino acid is then linked to the peptide chain held by the tRNA in the neighbouring P site. The large ribosomal subunit moves forward, moving the spent tRNA to the E site, and the small ribosomal subunit moves three nucleotides in the mRNA, bringing it back to the original position relative to the large ribosomal subunit. These movements eject the tRNA and resets the ribosome with a free A-site where the next tRNA can bind. These steps are repeated until a stop codon in the mRNA is met (UAA, UGA, UAG). The mRNA is translated in 5' to 3' direction, and the N-terminal of the protein is made first, which has the initiator amino acid methionine, with each cycle adding one amino acid to the C terminal of the protein [1][11].

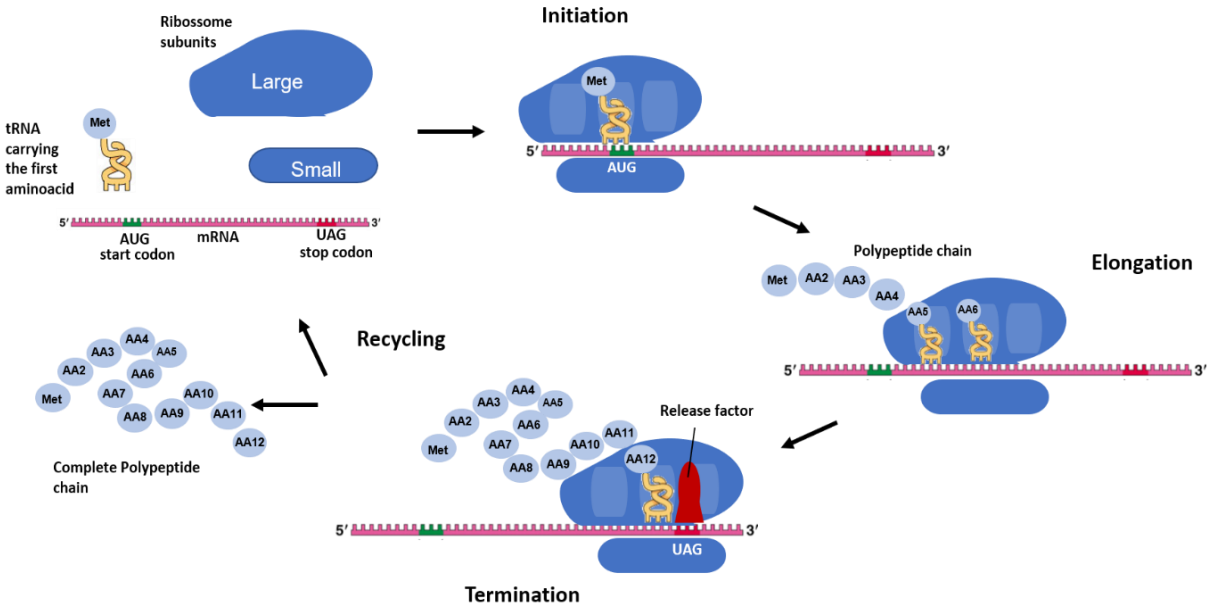

Figure 2- The protein synthesis mechanism involves three main stages, initiation, elongation and termination. During initiation there is the formation of a translation complex made of the ribosome subunits linked with the mRNA and the tRNA carrying the first amino acid, which binds to the start codon (AUG). During elongation, the tRNAs bring the amino acids corresponding to the mRNA's codons, forming a polypeptide chain. During termination, the stop codon (UAG) is recognized by a release factor and the translational complex disassembles. The polypeptide chain formed is released and the components of the translation complex are recycled.

1.2. mRNA Mistranslation

In protein synthesis, particularly in the translation process, cells must find an optimal balance between translational speed and accuracy, and therefore some level of errors in protein synthesis is tolerated to guarantee the speed of translation [2]. A gene is said to be mistranslated when the amino acid sequence synthetized doesn't correspond to the genetically encoded sequence [13]. These mistranslation errors can occur at any step that mediates the flow of information from the genetic code to proteins, however they're more common in translation than in transcription. DNA replication errors occur once in every 10^8 nucleotides, and they can result in amino acid identity change. On the other hand, nucleotide misincorporation during mRNA transcription is more common, at a rate of one in every 10^5 nucleotides [2]. This difference in the error rates between mRNA replication and transcription can be explain by the increased complexity of the protein synthesis process compared to the replication process [13].

Translation-associated errors can take place when an aminoacyl-tRNA synthetase (aaRS) charges the tRNA with a noncognate amino acid (aminoacylation errors), or during mRNA decoding by the ribosome. Aminoacylation errors can be due to failure of the aaRSs to recognize their cognate tRNA or by the activation of incorrect bound amino acids. To minimize such errors, aaRS have editing mechanisms that can take place before or after the transfer of incorrect amino acids to the tRNA. Pre-transfer editing mechanisms promote the hydrolysis of mischarged aminoacyl adenylates, whereas in post-transfer mechanisms the aminocyl-tRNAs are hydrolysed [2][14][15][16][13].

Errors associated with the mRNA's decoding process are mainly caused by codon's misreading (missense errors) and by readthrough of the stop codons (nonsense errors), which lead to synthesis of a mutant protein. Other types of errors include frameshift errors,

that alter the mRNA's reading frame and processivity errors, in which the ribosome terminates translation earlier, both producing truncated mutant proteins [14][15].

Although translation errors are inevitable, cells tolerate well some types of mistranslation and certain organisms maintain high levels of mistranslation. It's clear that mistranslation can have diverse effects, nevertheless the functional consequence of a certain translation error mainly depends on the cellular context. Thus, mistranslation is neither universally beneficial nor deleterious; it is a highly complex and variable process, that depending on the degree of errors, there may be different consequences at the molecular and cellular levels [17][2].

Unregulated mistranslation can make amino acid substitutions that generate mutant proteins, which are more prone to misfold and consequently form toxic protein aggregates [12]. The sticky mouse mutation is an example of the negative effects of unregulated mistranslation. A mutation in the editing domain of the alanyl-tRNA synthetase (AlaRS) leads to the charging of $tRNA^{Ala}$ with serine, resulting in the accumulation of misfold proteins and protein aggregation in mouse neuron cells [18][19].

On the other hand, cells must find a balance between translation speed and accuracy; thus, by allowing a certain degree of error, the rapidity of translation is guaranteed. Therefore, the most obvious benefit of mistranslation is the acceleration of protein synthesis. Another potentially beneficial effect of mistranslation is the activation of transcriptional stress responses, which can confer a wide tolerance to stressors. Mistranslation also seems to function as a mechanist that generates cell surface variation, and therefore may help organisms in evading the host immune response. Mistranslation is associated with proteome diversification, which can enhance protein function. Amino acid substitutions within proteins can result in novel protein phenotypes that may be beneficial [20].

There are several examples of organisms that maintain high levels of mistranslation due to it's beneficial effects. Mycoplasma spp. have point mutations and deletions in the editing domains of their ThrRS, LeuRS and PheRS, which decrease the fidelity of tRNA aminoacylation, resulting in high levels of mistranslation (figure 3A). In its turn, higher mistranslation levels are associated with a greater cell surface variability, which may help this parasite escape the host immune response. Another key example of ambiguous translation is the CUG decoding ambiguity in *Candida albicans* (figure 3B). Due to Ser-$tRNA_{CAG}$ being recognized by both SerRS and LeuRS, both leucine and serine can be incorporated at CUG codons. The main consequence of CUG decoding ambiguity is the

proteome expansion and consequently the phenotypic diversity generated, both of which are detailed in the next section [2].

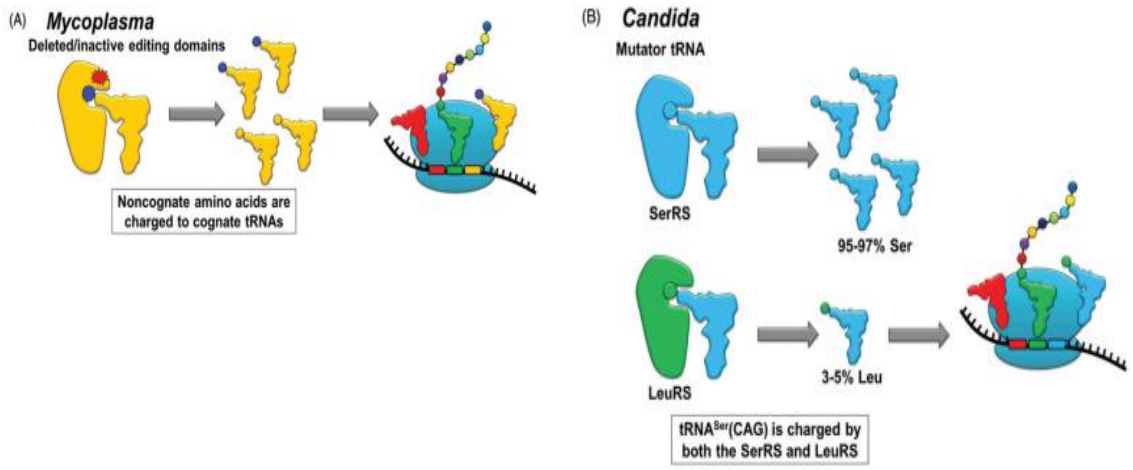

Figure 3- Naturally ambiguous translation. (A) Mycoplasma species have point mutations and deletions in the editing domains of several tRNA synthetases, which allow charging of noncognate amino acids and consequently reduces the fidelity of aminoacylation, resulting in high levels of mistranslation. (B) In Candida species the *Ser-tRNA*$_{CAG}$ is recognized by both SerRS and LeuRS and consequently both serine and leucine can be incorporated at CUG sites. Adapted from [2].

1.3 Ambiguous translation of the CUG codon in *C. albicans*

In most organisms, according to the genetic code, the CUG codon is translated as leucine. However, *C. albicans* has a unique genetic code, as the CUG codon is ambiguous and thus can be translated as serine, 97% of the time, or leucine, 3% of the time [21][22][23].

This ambiguous decoding process was originated from mutations on the ancestral Ser-tRNA, that produced a hybrid molecule of Ser-tRNA$_{CAG}$, which has the body of a Ser-tRNA and the anticodon of Leu-tRNAs (5'-CAG-3'). Thus, this molecule contains identity elements that are recognized by both the SerRS, (three G–C base pairs of the extra loop and the discriminator base G73) and also by the LeuRS (which recognizes the adenosine on A35 and the methyl group of mG37 on the anticodon loop). This enabled the formation of a Ser-tRNA that exists in two different forms: the Ser-tRNA$_{CAG}^{Ser}$ (charged with serine) and the Leu-tRNA$_{CAG}^{Ser}$ (charged with leucine). The existence of these two tRNAs generates ambiguity at CUG codons as they compete with each other for CUGs at the ribosome's A

site [24][23]. However, SerRS is the main charging enzyme of the Ser-tRNA$_{CAG}$ and the LeuRS is a poor competitor, resulting in the incorporation of both leucine (3%) and serine (97%) at CUG sites.

As serine is hydrophilic and leucine is hydrophobic, the change in identity of CUG codons results in altered protein structure and function [17][21][22]. Several studies have shown the phenotypic and proteome diversity generated by CUG ambiguity. In the work of Gomes et al, it was shown that an increase in ambiguity did not decrease growth rate and that *C.albicans* cells tolerate up to 28.1% Leu misincorporation. In fact, under stress, such as low pH, there's a slight increase in CUG ambiguity, that generates proteome diversity [22].

In the work of Miranda et al, it was shown that CUG ambiguity resulted in expansion of functional and structural diversity of cell surface proteins, creating cell surface variation. In fact, they showed that, the coding sequence of ALS genes, which are a family of genes that encode adhesins which bind to different substrates, is rich in CUG codons. Thus, mistranslation probably plays an important role in the interaction of *C.albicans* with the host via creating cell surface variation [25].

In the work of Bezerra et al, it was possible to construct a series of strains with different levels of Leu misincorporation (3-98%) by incorporating one or two copies of a mutant Leu tDNA$_{CAG}$ gene into the genome of *Candida albicans* SN148 strain, and deleting the endogenous Ser-tRNA$_{CAG}$. They showed that CUG ambiguity is a mechanism that generates phenotypic diversity, which allows the adaptation of *C.albicans* to different conditions and environmental pressures. Ambiguous cells were able to grow in different conditions, being tolerant to commonly used antifungal drugs, such as fluconazole. They showed that such tolerance involved mutations in antifungal target genes and multidrug transporters, and that unique SNPs accumulate in genes related to filamentous growth and cell adhesion, that are important virulence traits [26].

The work of Weil et al was crucial, as it demonstrated that mistranslation plays a key role in the development of drug resistance. Their data suggested that mistranslation was responsible for a faster acquisition of drug resistance through a range of mechanisms, and that hypermistranslating strains had a different and broader range of mutational and gene deregulation, such as multiple chromosome duplications, partial chromosome deletions and polyploidy [27].

1.4. Candida albicans Biology

Candida albicans is a diploid polymorphic fungus that was first described in 1839 [28]. It *b*elongs to the ascomycete phylum, which includes the model yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. This fungus exists as a commensal in the gastrointestinal tract of humans and other warm-blooded animals, and at the same time, it's the most common human fungal pathogen, causing mucosal and systemic infections [28][29][30]. One of the most significant features of *C. albicans* is its ability to grow in different shapes, being a morphologically complex organism [31]. It also has an extraordinary ability to adapt to different conditions and proliferate in different environments, which is closely associated with its pathogenicity [3].

1.4.1. Epidemology of Candida infections

C. albicans is a part of the normal human microbiome, colonizing the digestive, vaginal and oral mucosae in a non-harmful way, as the normal microbiome and the host immune system prevent it from causing disease. However, certain conditions can trigger infection, such as immune compromised hosts, microbiota imbalances or variations in the local environment [32][33][34][4]. Infections with *Candida spp.* are a big cause of morbidity and mortality and can be manifested as a dispersed systemic bloodstream infection (candidemia) or as a localized superficial mucosal infection [32][33]. *C.albicans* is the main cause of candidiasis, with up to 75% of all women worldwide are affected with vaginal candidiasis at least once in their lifetime, with recurrent forms in 5-10% of cases; and oropharyngeal candidiasis occurs in 90% of HIV-infected patients with AIDS. Candidemia infections are very serious conditions with mortality rates of about 30-50% These high mortality rates and morbility rates are associated to a poor diagnosis, to the reduced number of antifungal drugs available and to the increasing number of resistance to antifungal drugs [35][36]. During the infection process, *C. albicans* must face many different host environments, to which it must adapt in order to survive. It does so through a series of virulence factors and fitness attributes that promote the pathogenicity of *C.albicans* [33].

1.4.2. Morphology

One of *C. albicans* main characteristics it's the ability to switch between different morphologies yeast, pseudohyphae and hyphae, each one playing a role on the pathogen's colonization and surviving cycle [36][37]. This morphology diversity is a key feature of *C.albicans,* as it allows its survival, growth and dissemination in the host. All the three morphologies have a single nucleus before mitosis, however there are some important differences between them [37]. The yeasts are separated cells that have an ovoid shape; the hyphae consist of linked yeast cells, that form a tube-like structure with paralel sides, that is highly polarized and doesn't have any constrictions at septation sites; and the pseudohyphae are similar to hyphae but they have constrictions sites and they are wider [37][36]. The hyphal form is associated with tissue invasion, as hyphal cells express cell-wall proteins associated with adhesion to tissues; whereas the yeast form is involved in the dissemination of the pathogen in the bloodstream. Thus, the transition from yeast to hyphae, named dimorphism, determines *C.albicans* commensal and pathogenic states, and is required for its pathogenicity [37][20]. Several environmental conditions can trigger signal transduction pathways that induce this transition from yeast to hyphae (detailed in section 1.4.5).

1.4.3. Genome

C.albicans genome is organized in eight diploid chromossomes (1-7 and R) and has about 14 Mb (haploid) comprising around 6100-6200 genes [38][39]. An important feature of *C.albicans*'s genome is its plasticity, which is associated with the virulence and pathogenicity of this pathogen [39][20]. This plasticity includes genomic changes such as chromosome rearrangements, copy number variation, aneuploidy, and loss of heterozygosity. It is well described in the literature that many fungi, including *C.albicans*, undergo genomic changes in order to survive in stressful environments, such as when exposed to antifungal drugs [39].

Changes in chromosome size have been detected in *C.albicans*, and they are due to chromosome translocations, repeat length changes, truncations, telomere recombination events and supernumerary chromosome formation. Most of these chromosome changes involve major repeat sequence (MRS) as they allow recombination between nonhomologous chromosomes. In *C.albicans*, 9 MRS have been detected. On the other hand, supernumerary chromosomes are additional chromosomes, usually generated from

extra copies of other chromosomes. They have highly variable regions, high mutation rates and contain genes acquired through horizontal gene transfer, particularly genes associated with virulence and pathogenicity, which allows the fungi to colonize the host [39][40].

Cellular ploidy can be described as number of complete sets of chromosomes in a cell. It has been shown that fungi undergo ploidy changes during adaptation to new environments. Ploidy changes, by altering the rate and diversity of beneficial mutations, have an impact on the cell's ability to adapt to new conditions and stresses and thus play a role on the cell's survival. Aneuploidy is commonly seen in *C.albicans*. It is defined as the change in the total number of chromosomes such that one or more chromosomes are present in excess or are missing, and usually occurs due to chromosome nondisjunction during mitosis [41]. Aneuploidy of a specific chromosome can generate new phenotypes because of the copy number of specific genes on that chromosome. Thus, it's not aneuploidy but the copy number of specific genes that confers many of the aneuploidy-associated phenotypes that are seen. Some aneuploidies provide fitness benefits under specific conditions, functioning as a mechanism of generating diversity in a short period of time, which can be important for species that lack sexual reproduction, as in the case of *C. albicans*. Therefore, by generating diversity, aneuploidies allow the organism to adapt to new conditions and stressors, and can drive the selection process [41][40]. In previous works with the evolution of *C.albicans* in the presence of antifungal drugs, in this case flucanozole, there was an increase in the frequency of ploidy changes and subsequent aneuploidies due to chromosome gain or loss, and certain aneuploidies were advantageous and drove selection [41]. One example of an aneuploidy that arises during *in vitro* evolution of *C.albicans* with flucanozole is the aneuploidy of isochromsome 5L (i(5L)), that allows the amplification of two genes (ERG11 and TAC1). As ERG11 is the target of azole drugs and TAC1 is required for the up-regulation of the ABC drug transporters CDR1 and CDR2 the formation of (i(5L) is involved flucanozole resistance. Due to its high fitness benefit, it accumulates rapidly in the population. There are many other examples demonstrating that aneuploidies can lead to rapid adaptation to certain environments [40].

1.4.4. Parasexual cycle

Until recently it was thought that *Candida albicans* didn´t have a sexual cycle because of the inability of finding mating forms, being therefore classified as an asexual obligate diploid organism [28][29]. Currently it's known that this pathogen has a parasexual cycle (Figure 4) as it was detected a mating-type locus, MTL, on chromosome 5, which encodes proteins

similar to the mating locus MAT in *S. cerevisiae* [38][31]. The MAT locus in *S. cerevisiae* contains two alleles a and α, that determine the mating type. a-type cells can only mate with α-type cells, producing an heterozygous diploid that is unbale to mate, and instead undergoes meiosis and generates haploid cells type a and α, which are able to mate [28]. *C. albicans*'s MTL is very similar to *S. cerevisiae*'s MAT as it also contains the two alleles a and α. However, unlike in *S. cerevisiae* and other fungi, mating in *C. albicans* is directly linked to phenotypic switching between two states morphologically different, white and opaque. White cells are rounder and form dome-shaped colonies on agar, while opaque cells are more elongated and form flatter colonies on agar. This ability to switch between different shapes was found to be regulated by proteins encoded by the MTL loci. As opaque cells were shown to mate more efficiently that white cells, cells must undergo a transition from white to opaque in order to be able to mate efficiently [28][29][30].

In the parasexual cycle of *C. albicans*, diploid cells (2n) are normally heterozygous at the MTL and undergo loss of heterozygoty of one copy of the MTL locus, eliminating an a or α copy, producing a/a or α/α diploid strains (2n). These MTLa and MTL α strains must switch from white to the opaque state. Diploid MTLa and MTLα opaque cells mate efficiently, creating tetraploids (4n) aa/αα that can either grow stably or undergo a concerted chromosome loss to return to diploidy (2n) a/α) [28]. Recently, haploid *C.albicans* cells have been isolated, which further emphasizes the plasticity of *C.albicans* genome [42].

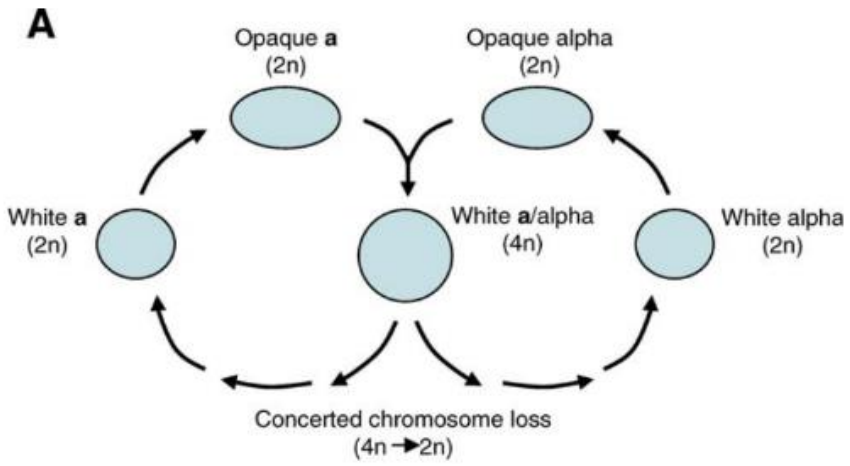

Figure 4- *Candida albicans* parasexual cycle. White MTLa and MTLα switch to the opaque state before mating and formation of a mononuclear tetraploid a/ α. Then by random chromosome loss, there is a reduction in ploidy back to diploidy. Adapted from [3].

1.4.5. Virulence

C.albicans is an opportunistic commensal yeast and therefore, when certain conditions are met, such as host immune suppression, it colonizes and invades host tissues. The ability to colonize host tissues is due to virulence factors, which are different mechanisms of the pathogen that enable it to colonize and invade tissues [3]. The most relevant ones are the transition between different morphologies, expression of adhesins and invasins on the cell surface, thigmotropism, formation of biofilms, phenotypic switching and secretion of hydrolytic enzymes (Figure 5). Other important factors are fitness attributes, which include adaptation to variations in the environment's pH and robust nutrient acquisition and stress response systems [3].

Polymorphism

Several environmental conditions trigger the transition of yeast cells to hyphae and pseudohyphae, in a process named dimorphism. Some of these conditions include temperature (temperatures above 37ºC induce hyphal formation), starvation, presence of serum and N-acetilglucosamine, pH (in environments with a pH bellow 6, the cells grow as yeasts, while at pH higher than 7, hyphal growth is stimulated) and CO_2 levels [37][3][36][4]. These conditions induce the expression of hyphal-specific genes (HSGs), whose expression is tightly regulated. Amongst the HSGs are genes encoding cell wall proteins (HWP1), adhesins (ALS3 and ALS8) and proteins required for invasive growth (RBT1) [37].

Another aspect that influences the transition from yeast to hyphae is the interaction of *C. albicans* with the microbiological flora in its environment. Through a quorum sensing mechanism, *C. albicans* detects the density of the surrounding population, based on the production and release of farnesol, which induces growth in the yeast form. Thus, high cell densities are associated with higher farnesol levels, inducing yeast formation, whereas low cell densities are associated with low farnesol levels and thus, hyphal formation [36].

Adhesins and invasion

Adhesins are a specialized group of proteins that induce attachment of *C. albicans* to other microorganisms or to host tissues, and therefore are important for the process of host colonization. This colonization can occur through two different mechanisms: endocytosis, in which the pathogen expresses cell surface proteins that bind to host ligands, allowing the

entrance of the fungus into the host; and active penetration of the plasma membrane, mediated by hyphal extension and by the secretion of hydrolases. The hydrolases facilitate nutrient acquisition and can be classified as proteases, lipases and phospholipases [3][36][43].

Thigmotropism

C. albicans cells have the ability to sense and respond to changes in surfaces, in a process called thigmotropism. In this case, upon contact with a surface, hyphal growth is induced. The hyphal cells recognize the substratum and respond accordingly. When in contact with solid surfaces, biofilm formation is triggered, and in certain substrates like agar or mucosal surfaces, the hyphae penetrate into the substrate [3][44].

Biofilms

A biofilm is a community of microorganisms that adhere to a surface and are surrounded by extracellular matrix, having different properties from the floating, planktonic cells. The biofilm structure provides a stable environment for microbes, being much more resistant to physical and chemical stressors than free planktonic cells [45]. The majority of diseases triggered by *C.albicans* involve biofilm formation on host or abiotic surfaces. Thus, the ability to form biofilms on different surfaces is a key characteristic of this pathogen. Such surfaces include host tissues or abiotic surfaces like urinary and central venous catheters, pacemakers, mechanical heart valves, contact lenses, etc [45][4][46]. Biofilm formation in these medical devices is a threat to human health as the *Candida spp*. biofilm can disseminate in the bloodstream, leading to systemic infections of tissues and organs. Candida's biofilms are highly structured and are composed of multiple cell types (pseudohyphae, hyphae, yeast cells) encased in an extracellular matrix [46][4][45]. The process of biofilm formation involves different stages: yeast cells adherence to a surface, proliferation of yeast cells, formation of hyphal cells in the upper layer of the biofilm, production of extracellular matrix and dispersion of yeast cells from the biofilm [3][4]. The majority of *C.albicans* biofilms are resistant to antifungal drugs, making these infections difficult to treat.

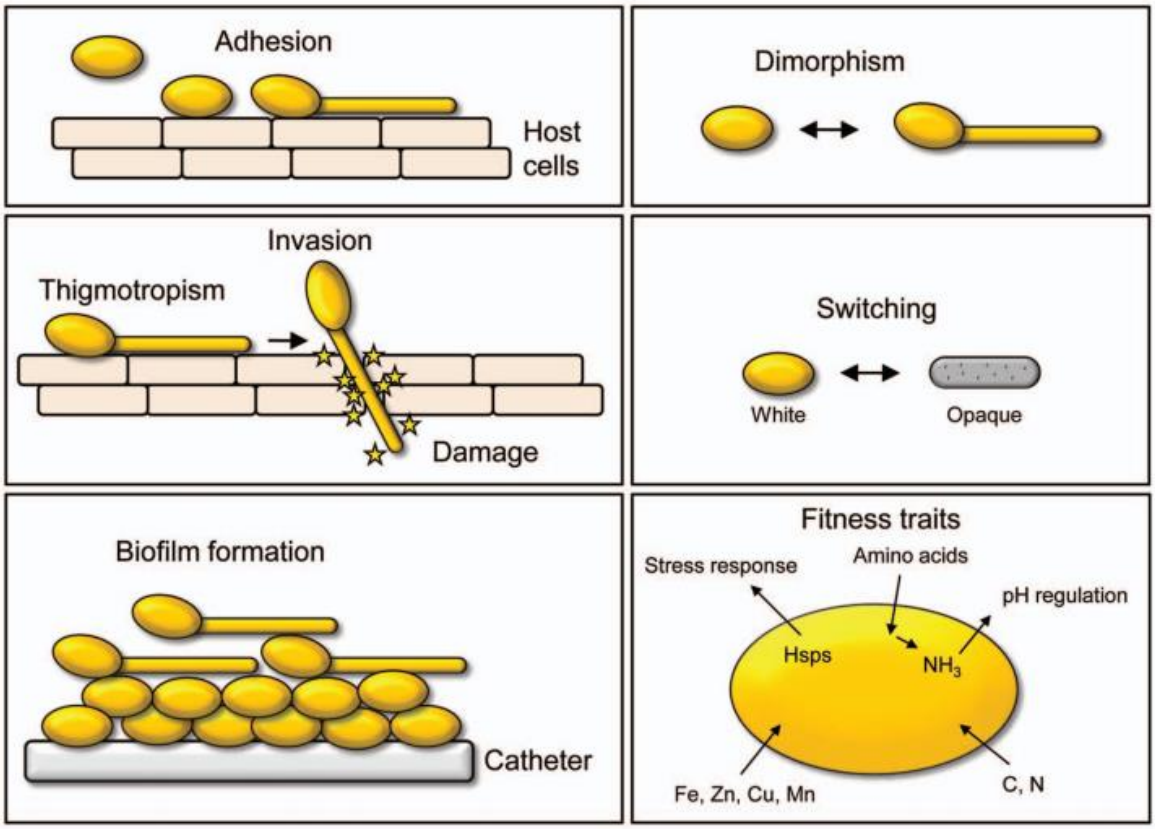

Figure 5- Overview of *C.albicans* virulence factors responsible for its pathogenicity. Adapted from [4].

1.5 Antifungal drugs and Resistance

1.5.1 Mechanism of Action of Antifungal drugs

Fungal infections are associated with several diseases, including acute diseases such as cryptococcosis and invasive aspergilosis, chronic diseases like allergic broncopulmonary aspergilosis, and superficial infections such as vaginal and oral candidiasis. These conditions are more prone to take place in an immunocompromised host [47]. The emergence of fungal infections has led to an increase in the use of antifungal agents for the treatment and prevention of these infections, which in turn is associated with the development of drug resistance [48] [47]. As fungal pathogens and human hosts are both eukaryotes, they are evolutionary similar, and consequently the drugs that specifically target fungi are very limited [49][50]. Thus there are very few classes of antifungal drugs available,

namely the azoles, polyenes, echinocandins, nucleoside analogs and allylamines (Figure 6) [48].

The azoles are the most widely use class of antifungal drugs, and they contain a 5-member azole ring attached to a side chain. They are classified in two types, imidazoles and triazoles, based in the number of nitrogen atoms in the azole ring, two nitrogen atoms in imidazoles and three in triconazoles. The imidazoles include ketoconazole, miconazole, econazole, tioconazole and clotrimazole, and the triazoles include fluconazole,itraconazole, voriconazole and posaconazole [51]. The azoles inhibit ergosterol biosynthesis by interfering with the cytochrome P450-dependent enzyme lanosterol 14-a-demethylase, encoded by ERG11 in yeasts. This enzyme is involved in the conversion of lanosterol into ergosterol, an important component of the fungal cell membrane. The inhibition of lanosterol 14-a-demethylase leads to a depletion of ergosterol and accumulation of toxic sterol precursors, resulting in altered membrane structure and function, which consequently inhibits fungal growth (Figure 7) [51][48][6].

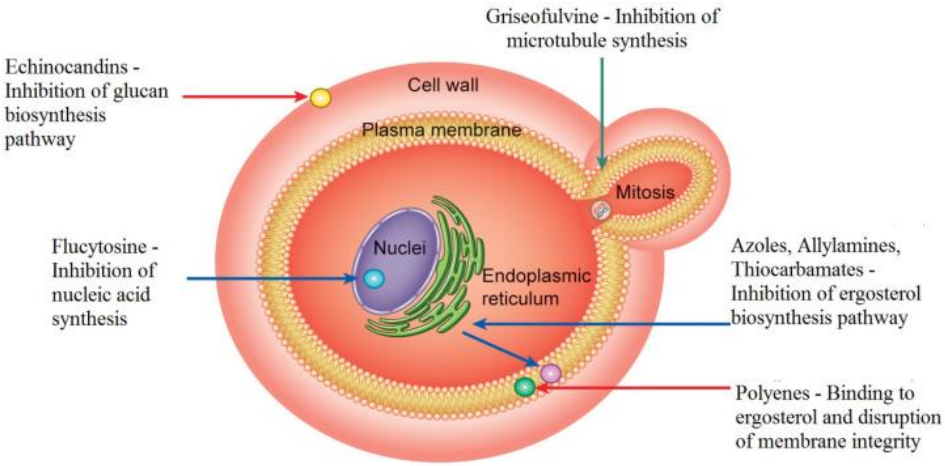

Figure 6-.Mechanisms of action of antifungal agents on cellular targets. Adapted from [5].

The mode of action of the polyenes, such as amphotericin B and nystatin, also involves interference with ergosterol. In this case, these compounds bind to ergosterol, promoting the formation of concentration-dependent channels that allow of extravasation of intracellular constituents, leading to cell death. However, amphotericin B exhibits high toxicity in the host, probably due to interference with host-cell membranes containing cholesterol [52][49][51].

The echinocandins are the newest class of antifungals, and they include caspofungin, micafungin, and anidulafungin. They are lipopeptides that inhibit the enzyme (1,3)-b-D-glucan synthase, which is a key element in cell wall biosynthesis, encoded by the FSK1 gene (FSK2 in *Candida glabrata*). This inhibition prevents glucan synthesis, thereby decreasing the levels of glucan polymers in fungal cells and resulting in an unstable cell wall that can't endure the osmotic stress. As animal cells don't have a cell wall, the use of echinocandins is associated with lower risk of side effects [51][48].

The allylamines act by inhibiting the early steps of ergosterol biosynthesis, namely the enzyme squalene-epoxidase, that plays a role in the synthesis of ergosterol, being encoded by the ERG1 gene. This inhibition leads to the accumulation of the ergosterol precursor squalene and the absence of intermediate sterols. The accumulation of squalene increases membrane permeability and consequently promotes membrane rupture [51][6].

Nucleoside analogs include the drug flucytosine (5-FC), which is a fluorinated pyrimidine that exhibits inhibitory activity against many yeasts. After entering the fungal cell,5-FC inhibits the enzyme thymidylate-synthetase, which is involved in DNA synthesis and nuclear division. 5-FC can also be converted in 5-fluorouacil and further phosphorylated to 5-flourodeoxyuridine monophosphate. The phosphorylation of 5-flourodeoxyuridine monophosphate allows it to be incorporated in RNA molecules, which interferes with the translation process. 5-FC is normally used together with amphotericin B due to its strong side effects, such as hepatic damages, interference with the bone marrow function and rapid development of resistance, especially among *Candida* species [51][6].

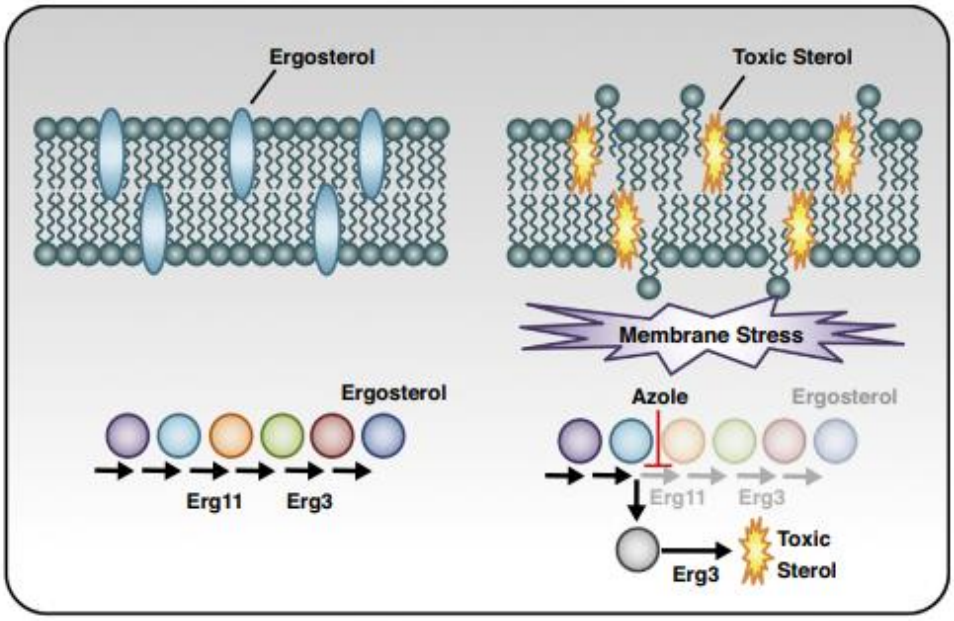

Figure 7- Mode of action of the azole drugs. Adapted from [6]

1.5.2. Mechanism of Drug Resistance

Drug resistance is an evolutionary process based on the selection of organisms with a better ability to survive and reproduce in the presence of drug. In this selection process, microorganisms develop strategies to outcompete their neighbours [49]. Drug resistance is not only a major threat to human health but also has major economic consequences.

Besides the capability of acquiring resistance through genomic alterations, some fungal species are inherently resistant to antifungals. Thus, resistance to antifungal drugs in fungi can be divided in primary and acquired resistance. Primary or intrinsic resistance exists prior to antifungal exposure, being used to describe isolates with innate resistance to antifungal drugs. On the other hand, secondary or acquired resistance takes place after antifungal exposure. Multiple factors influence and enhance acquired resistance in fungal pathogens, such as the organism's genetic plasticity, the existence of hypermutator strains and environmental pressures that favour the development of resistance [53][5][51]. Various adaptative mechanisms of antifungal drug resistance have been identified, such as drug target alteration or overexpression, upregulation of multidrug transporters and activation of stress response signalling (figure 8) [6].

Among the antifungal drugs available, members of the azole class, particularly fluconazole, have been the prime choice for treatment of invasive fungal disease due to low cost and

toxicity, accessibility and oral bioavailability [51][50]. However there are increasing cases of drug resistance reported in the literature, specially concerning the azoles class [51]. In fact, the most effective antifungal agents are fungistatic and therefore do not destroy the fungus but instead they stop its growth, as is the case of fluconazole. This allows the fungus time to develop resistance to the antifungals by becoming able to replace the fungal proteins destroyed by the drug or to quickly remove the drug from the cell [54].

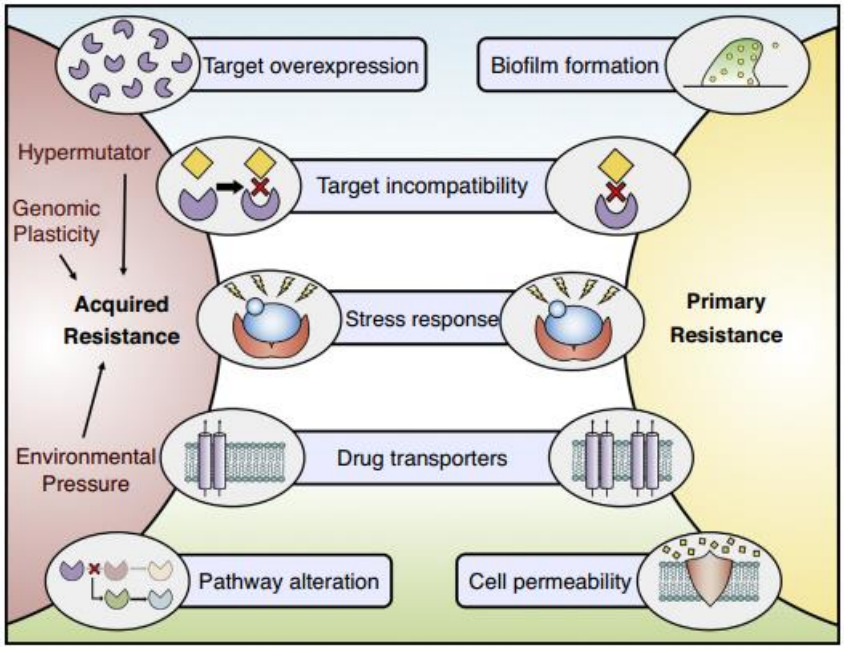

Figure 8- Mechanisms behind acquired and primary resistance. Adapted from [6].

Mechanism of Azole resistance

The main target of azoles is the cytochrome P450-depended enzyme lanosterol 14-a-demethylase, encoded by the ERG11 gene in yeasts (which is homologous to Cyp51A in molds). There have been identified four major azoles resistance mechanisms in *Candida spp*: alteration of drug target, overexpression of drug target, decreased intracellular drug levels and development of bypass pathways [5] [54] [47].

A common mechanism of azole resistance is through amino acid substitutions in the drug target, which decreases the affinity of the drug for its target and consequently prevents drug

binding. This mechanism is quite common in *Candida* species, as there have been identified over 140 amino acid substitutions [55][47]. Additionally, alterations in other components of the ergosterol pathway, such as loss-of-function of the D-5,6- desaturase enzyme ERG3, can also promote drug resistance. Mutations in ERG3 lead to the depletion of ergosterol and the accumulation of alternative toxic sterols, which often results in cross-resistance to azoles and polyenes [48].

Another azole-resistance mechanism is the amplification of the drug target, in this case the overexpression of ERG11, which minimizes the impact of the drug on the cell. The overexpression of ERG11 can occur by duplication of the left arm of chromosome 5 (i(5L)), which is a common aneuploidy in *C.albicans*. Gain-of-function mutations in the transcriptional activator Upc2, which regulates most of the ergosterol biosynthesis genes, also results in the overexpression of ERG11 [48] [47] [49] [55].

Active efflux of the drug through the activation of membrane-associated multidrug transporters is another important mechanism of azole resistance [6][48][47]. In fungi there are two main efflux systems, the ATP-binding cassette (ABC), encoded by CDR1 and CDR2, and the major facilitator superfamily (MFS), encoded by MDR1. Overexpression of these efflux proteins reduces the intracellular levels of drug, and has been reported as the major cause of azole resistance in *Candida albicans* [47][6].

In addition to the mechanisms described above, *Candida albicans* developed stress response strategies that allow cells to cope with several environmental stressors, like oxidative stress, osmotic stress, thermal stress, changes in pH and nutrient limitation. These pathways are critical for fungi to survive the stress induced by antifungal agents [56][57]. A key regulator of stress responses in all eukaryotes is Calcineurin, which is a Ca^{2+}-calmodulin-activated protein phosphatase that plays an important role in several fungi physiological processes, such as cell cycle progression, morphogenesis, virulence and antifungal drug response. In *Candida albicans*, this protein is critical for this pathogen's survival during the membrane stresses induced by fluconazole [58]. The transcription factor Crz1 is a downstream effector of calcineurin, and has been associated with azole resistance in *Candida albicans*, as it induces the expression of genes involved in cell wall integrity [59][60].

Another important player in the cell-stress response is Hsp90, which is a molecular chaperone that regulates the form and function of various signal transducers by enabling specific cell signalling pathways required for cells to survive the membrane stress induced by the antifungal drug. Hsp90 is also involved in the development of azoles resistance

through the loss-of-function of ERG3 [47][49]. Furthermore, this chaperone also interacts with the catalytic subunit of calcineurin, keeping it in a stable conformation required for activation [61][57]. In fact, the inhibition on calcineurin function is phenotypically similar to the inhibition of Hsp90, eliminating azole resistance in several mutants. Therefore, calcineurin is a key mediator of Hsp90-dependent azole resistance [57].

Resistance to Polyenes

For a long time, amphotericin B was the standard therapy for systemic fungal infections. It is a fungicidal agent that interacts with membrane sterols and creates aqueous that alter the membrane stability and lead to cell death [6][5][52]. There has been identified resistance to polyenes in *Candida* isolates, which is associated with mutations on components of the ergosterol biosynthesis pathway, like ERG2, ERG3 and ERG11.This mechanism of resistance involves a decrease in the ergosterol content and accumulation of alternative sterols. In fact, cross-resistance between azoles and amphotericin B has been seen in *C.albicans* isolates with mutations in ERG3, and also in *C.tropicalis* isolates with mutations in ERG11 and ERG3 [58].

Resistance to Echinocandins

The echinocandins are the newest class of antifungal drugs released into the clinic. A common mechanism of resistance to echinocandins in fungi is through alterations of drug target by mutations on the FSK1 gene, namely amino acid substitutions in conserved regions of the FKS subunits of glucan synthase [48][55][49]. *Candida* isolates resistant to echinocandins often have mutations in these hot spot regions [56].

It's been shown that calcineurin also plays a role in echinocandin resistance. In fact, the impairment of calcineurin's function decreased *Candida albicans's* tolerance to echinocandins. As calcineurin is associated with echinocandin resistance and Hsp90 regulates calcineurin's stability and function, Hsp90 also plays a role in responses to echinocandins. In fact, the inhibition of Hsp90 decreases echinocandin resistance in *Candida albicans* and results in a fungicidal combination. Thus, cell wall signalling mediated through protein kinase C (PKC), calcineurin and Hsp90 is crucial for development of echinocandins resistance [57].

1.6. Objectives

Candida albicans is a pathogenic fungus responsible for serious infections in humans, especially in immunocompromised hosts, being associated with high mortality and morbidity rates [15][62][16][11]. Recently, with the emergence of fungal infections, there has been an increase in the development of resistance to antifungal agents [63][43].

Previous studies showed that mistranslation in *C.albicans* can be an advantageous process that results in phenotypic diversity and genomic plasticity. One of the phenotypes observed in hypermistranslating strains was their tolerance to antifungals, particularly fluconazole [26][64][27]. Although the mechanism is still unclear, we hypothesized that mistranslation accelerates the acquisition of resistance to fluconazole through genomic alterations. To test this hypothesis, we addressed the following specific objectives:

1. *In vitro* evolution of hypermistranslating and wild-type strains in the absence and presence of fluconazole;
2. Evaluate the effect of mistranslation on the acquisition of resistance to fluconazole;
3. Measure mistranslation levels during the evolution of drug resistance;
4. Sequence the genome of hypemistranslators evolved *in vitro* to clarify the mechanisms of fluconazole resistance

2 Material and Methods

2.1. Strains and Growth conditions

This study used *C. albicans* strains T0, T1 and T2, which were engineered by Bezerra and colleagues and exhibit different levels of leucine misincorporation at serine CUG codons (Table 1) [26]. *C.albicans* strains T0, T1 and T2 were grown at 37ºC in Yeast Peptone Dextrose (YPD), containing 2% glucose, 1% yeast extract and 1% peptone.

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

Strains	Genotype	%mistranslation
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%

2.2. Experimental evolution

18 clones of each strain (T0, T1 and T2) were subjected to an *in vitro* experimental evolution (Figure 9 and table 2). Two different conditions were tested: the evolution of the *C.albicans* strains with and without fluconazole. Each clone was inoculated in YPD media in a 96-well plate kept at 37ºC. Every two days an aliquot of stationary phase culture was transferred to fresh media. In each passage, the fluconazole concentration of the liquid growth medium

was adjusted to double the concentration of the last MIC (minimal inhibitory concentration) measured. Passages were pursued until clones were growing in liquid media containing a fluconazole concentration of 256 µg/ml. The same number of passages (10 passages corresponding to approximately 200 generations) were pursued for the strains growing without drug. All clones were frozen in 80% glycerol.

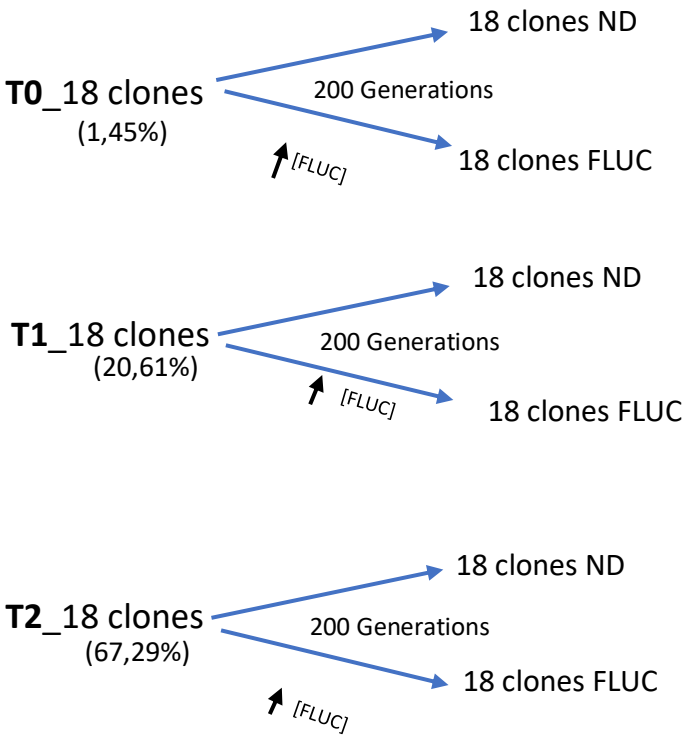

Figure 9- Scheme of the experimental evolution performed.

Table 2- *C.albicans* clones used in this study.

Strain	Clone number	Clone name	Strain	Clone number	Clone name	Strain	Clone number	Clone name
	1	T0_UUA1		1	T1_UUA1		1	T2_UUA1
	2	T0_UUA2		2	T1_UUA2		2	T2_UUA2
	3	T0_UUA3		3	T1_UUA3		3	T2_UUA3
	4	T0_UUA4		4	T1_UUA4		4	T2_UUA4
	5	T0_UUA5		5	T1_UUA5		5	T2_UUA5
	6	T0_UUA6		6	T1_UUA6		6	T2_UUA6
	7	T0_CUG1		7	T1_CUG1		7	T2_CUG1
	8	T0_CUG2		8	T1_CUG2		8	T2_CUG2
T0	9	T0_CUG3	T1	9	T1_CUG3	T2	9	T2_CUG3
	10	T0_CUG4		10	T1_CUG4		10	T2_CUG4
	11	T0_CUG5		11	T1_CUG5		11	T2_CUG5
	12	T0_CUG6		12	T1_CUG6		12	T2_CUG6
	13	T0_UCU1		13	T1_UCU1		13	T2_UCU1
	14	T0_UCU2		14	T1_UCU2		14	T2_UCU2
	15	T0_UCU3		15	T1_UCU3		15	T2_UCU3
	16	T0_UCU4		16	T1_UCU4		16	T2_UCU4
	17	T0_UCU5		17	T1_UCU5		17	T2_UCU5
	18	T0_UCU6		18	T1_UCU6		18	T2_UCU6

2.3. Determination of minimal inhibitory concentration (MIC)

In order to examine the development of resistance to fluconazole, the European Committee on Antimicrobial Susceptibility Testing (EUCAST protocol) was used. This method is based on the preparation of working solutions of antifungal agents in 100µL volumes/well, to which 100µL of inoculum are added. 96-well plates were prepared consisting of a series of wells with YPD with increasing fluconazole concentrations (0.125-256 µg/mL range), and wells without the drug (control wells). Plates were inoculated with a standardized number of cells of each clone of each strain (1×10^7 cells counted using the TC10^{tm} Automated cell counter from BioRad). The microdilution plates were incubated for 24h at 37ºC without agitation. Then, the absorbance was read at 595 nm using the iMARK^{tm} Microplate Reader. The value of the MIC for each passage 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.

2.4. Microscopy

To evaluate if there was any difference in the morphology between the T0, T1 and T2 strains evolved with and without fluconazole, cells of 2 clones of each strain were photographed using the Zeiss MC80 Axioplan2 light microscope. Cells were grown until exponential phase and were then poured onto a microscope slide previously coated with a bed of 1% agarose. Microscope fields were randomly chosen and at least 100 cells were analysed per sample. Photographs were taken using an AxioCamHRc camera.

2.5. Quantification of leucine mistranslation

Levels of mistranslation at the beginning and end of the evolution experiment were measured using a gain-of-function reporter system based on the yeast enhanced green fluorescent protein (yEGFP), developed by Bezerra and colleagues [26]. This reporter system only becomes active (expresses active GFP) when leucine is incorporated at codon 201 (Leu201). If serine is incorporated at codon 201 (Ser201), the GFP reporter is inactive.

The Leu UUA codon at position 201 is a positive control that always incorporates leucine and emits fluorescence, while the Ser UCU codon at position 201 is a negative control that always incorporates serine and doesn't emit fluorescence. The ambiguous CUG codon can incorporate both leucine and serine at codon 201. This allows the quantification of the mistranslation rate since the fluorescence emitted will be proportional to the insertion of leucine at that position.

In our experiment, each strain was represented by 6 clones containing the different versions of the reporter system (6 clones Leu-UUA201, 6 clones Ser-UCU201 and 6 clones Leu/Ser-CUG201) (Table 2). For each clone of each strain, yEGFP expression was detected by flow cytometry. Clones were grown overnight at 37ºC in liquid medium until reaching an OD_{600nm} of 0.8-2.0 and aliquots were filtered using the sterile Cell Strainer (40 μm Nylon Mesh from Fisher) and analysed using the flow cytometer BD Accuri C6.

Analysis was performed based on light-scatter and fluorescence signals. Signals corresponding to forward angle and 90º-side scatter (FSC and SSC) and fluorescent signals were documented. The fluorescent signals were screened using the FL1 filter, a 530nm

band-pass filter for GFP. 20.000 events were recorded at a slow flow rate. The data acquired was analysed with the Accuri C6 Sampler software. After obtaining the values of intensity of fluorescence of clones UUA201 (positive control) and Ser/Leu-CUG201 (reporter), the value of fluorescence of the clones Ser-UCU201 (negative control) was subtracted and the ambiguity level was obtained as a ratio between CUG201 and UUA201.

2.6. Ploidy determination by flow citometry

Flow cytometry was used to determine the ploidy of *C.albicans* cells. To determine the effects of the experimental evolution on the DNA content of cells, all eighteen clones from strains T0, T1 and T2 were tested at initial and final passages (P0 and P10 respectively) of evolution with and without fluconazole. Two control samples were also included: strain SN148 was used as diploid control (2N) and an unstained control sample, which received the same treatment but wasn't stained with propidium iodide (IP).

Clones were grown overnight at 37ºC in liquid medium until an OD_{600nm} of 0.8-2.0. Aliquots were collected and after being washed with PBS 1x (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4), cells were fixed with ice cold ethanol (-20ºC) and incubated overnight at 4ºC. After being washed with PBS 1x, cells were incubated for 1h at 50ºC with RNAse A (Qiagen, 100 µg/µl), and then propidium iodide (1 mg/ml) was added to stain the cells. *C.albicans* cells were then incubated overnight in the dark at 4ºC and analysed with the flow cytometer BD Accuri C6 after filtering.

Analysis was performed based on light-scatter and fluorescence signals. Signals corresponding to forward angle and 90º-side scatter (FSC and SSC) and fluorescent signals were documented. Propidium iode staining was monitored in the FL2 channel (488nm). 20.000 events were recorded at a slow flow rate. The data acquired was analysed with the Accuri C6 Sampler software. Mean fluorescence intensities of cells in G1 and G2 phases of the cell cycle were quantified in control (2N SN148) and test samples.

2.7. Whole genome re-sequencing

2.7.1 DNA extraction

DNA was extracted from 3 different clones of strain T1: one clone with low MIC (0.125 µg/mL) from the initial passage (P0); one clone from the final passage of the evolution (P10) with drug with high MIC (256 µg/mL); and one from the final passage (P10) of evolution without drug, with low MIC (0.125 µg/mL) (table 3). The protocol used was an adaptation of the MasterPure Yeast DNA Purification Kit from Lucigen. 10mL of cells from a mid-log liquid culture (OD 1.0-1.5) were centrifuged for 10 minutes at 5000g, 4ºC and washed two times with 1mL of TE1x (10 mM Tris-HCl, pH 8.0; 1mM EDTA, pH 8.0). Then, cells were incubated for 3h at 50ºC with a mixture of Yeast Cell Lysis Solution and RNAse A (100 µg/µl). After, MPC Protein Precipitation Reagent was added and samples were centrifuged twice, with the supernatant being transferred to a new Eppendorf each time. Samples were centrifuged after adding isopropanol and then washed two times with EtOH 70%. DNA was suspended in TNE Buffer for 3h at 4ºC and then incubated overnight at 37ºC with RNAse A (100 µg/µl) and RNAse I (3 U/µl). After incubation, DNA was incubated on ice for 5 minutes with Yeast Lysis Solution. MPC Protein Precipitation Reagent was added and samples were centrifuged twice, with the supernatant being transferred to a new Eppendorf each time. Samples were centrifuged after adding isopropanol and then washed two times with EtOH 70%. After removing all the EtOH, the DNA was suspended in mQH2O at 4ºC overnight. The quality and quantity of the DNA was assessed using the Qubit fluorometric method, by measuring the A60:A280 and A60:A30 ratios in the spectrophotometer and by running the DNA samples in a 1% agarose gel.

2.7.2 Library preparation

Following DNA extraction, DNA libraries were prepared using the Nextera™ DNA Flex Library Prep protocol by Illumina. One library per sample was prepared with as insert size of ~600bp. First, DNA was diluted in mQ H2O to have a DNA input of 150ng. The first step of the protocol is the Tagmentation reaction, which fragments and tags the DNA with adapter sequences. Samples were incubated in the BioRad thermal cycler for 15 minutes at 55ºC, with a mix of BLT (Bead-Linked Transposomes) and TB1 (Tagmentation Buffer 1). After that, TSB (Tagmentation Stop Buffer) was added to stop the tagmentation reaction and samples were incubated for another 15 minutes at 37ºC. The next step is a clean-up to wash the adapter-tagged DNA before performing PCR amplification. To do so, samples

were transferred to a plate that was placed on a magnetic stand and the supernatant was discarded. Then, samples were washed two times with TWB (Tagment Wash Buffer), and afterwards, beads were resuspended in TWB. The following step is the amplification of the tagmented DNA. After discarding the supernatant, a PCR master mix (EPM -Enhanced PCR Mix) and water were added to the samples along with the index adapters. The PCR program ran on the Biorad Thermal Cycler using the program described on the protocol. After the PCR cycle, the amplified libraries were purified. The supernatant was removed and transferred to a new plate, using the magnetic stand. Diluted SPB (Sample Purification Beads) was added to the samples and the supernatant was transferred to a new plate, to which was again added SPB. The supernatant was removed and samples were washed twice with EtOH 80%, which was carefully removed. Then, beads were resuspended in RSB (Resuspension Buffer) and the supernatant was transferred to a new plate. The last step is checking the quantity and quality of the DNA libraries. To do so, libraries were quantified using the Qubit fluorimeric assay and were ran on the Agilent 2100 Bioanalyser to obtain the library size profile. Lastly, a Real-time PCR was done to quantify the libraries with more precision. This PCR uses 6 standards of known concentrations (20pM, 2pM, 0.2pM, 0.02pM, 0.002pM and 0.0002pM) to build a calibration curve, and then inserts our samples in the curve.

2.7.3 Sequencing

DNA libraries were sequenced using the MiniSeq plataform by Illumina following the manufacturer's guide protocol. Before loading, samples were diluted to 1μM and denatured with 0.1N NaOH. Denatured libraries were further diluted to 1.8pM with HT1 (Hybridization Buffer). For quality control, PhiX was used. Libraries were then loaded in the reagent cartridge and after checking the run and system status, the run was started.

2.7.4. Bioinformatic analysis

The sequencing run generates "fastq" files for each sample that were subjected to a bioinformatic pipeline. First, run quality control and pre-processing was performed. Raw quality and bias assessment was done with FastQC v0.11.7. Adapters and low quality bases were removed with Trimmomatic v0.36. Reads were mapped to the *C.albicans* reference genome, using the C.albicans SC5314 strain as the reference, downloaded from

the Candida genome database (www.candidagenome.org, assembly 21, allele A), using BWA 0.7.17. Base calibration and variant calling was performed with GATK v4.0.11.0, using the following filters: for SNP (QD<2.0; FS>60.0; MQ<40.0; MQRankSum < -12.5; ReadPosRankSum < -8.0), for INDEL (QD < 2.0; FS > 200.0; ReadPosRankSum < -20.0). Variants were annotated with SnpEff v4.3p and the final vcf (variant call format) file was generated. SNPs and INDELs were further filtered as in Selmecki et al, 2015: if DP < 10, SNP/INDEL were discarded; if 10 ≥ DP ≤ 20, at least 3 reads supporting an alternative allele are required to keep the alteration; if DP > 20, at least 5 reads supporting an alternative allele (DP = depth) [65]. Afterwards, a list of genes with missense SNPs within the coding sequence and other with the intergenic INDELs was obtained for each sample and the GO Slim Mapper tool in Candida Genome Database was used for functional enrichment.

3 Results

1. Analyse the effect of mistranslation on the acquisition of resistance to fluconazole

The emergence of fungal infections has led to the widespread use of antifungal drugs, which in turn leads to the appearance of resistant *C.albicans* strains [48][47]. These represent a threat to public health due to high mortality rates and high costs associated with treatment. This emphasizes the importance of understanding the mechanisms behind acquisition of resistance to antifungal drugs.

In order to analyse the effect of mistranslation on the acquisition of fluconazole resistance, we measured the ability of *C.albicans* to adapt to inhibitory concentrations of drug. We used strains constructed by Bezerra and colleagues, strains T0, T1 and T2 that have different levels of leucine misincorporation at CUG sites: 1.5%, 20% and 67% respectively [26]. Eighteen clones of each strain were grown in YPD media with and without fluconazole. In each passage, the fluconazole concentration of the liquid growth medium was adjusted to double the concentration of the last MIC measured. Experimental evolution was performed until cells reach 200 generations. Resistance to antifungal drugs was monitored using the European Committee on Antimicrobial Susceptibility Testing (EUCAST protocol). The value of the MIC established at the beginning and end of the evolution corresponds to the lowest concentration of fluconazole necessary to inhibit the growth of 50% of the population when compared with the drug free control.

Parameters used to determine susceptibility in resistant phenotypes were established according to European guidelines [66]. Strains able to grow with a concentration of fluconazole above 64 µg/ml are considered resistant, while strains that are not able to grow with a concentration bellow 8 µg/ml are considered susceptible.

The results presented in figures 10, 11 and 12 show the MIC values of 18 clones of strains T0, T1 and T2, in the end of the evolution. In the beginning of the experimental evolution (figures 10A, 11A, 12A), all clones from all strains were susceptible to the drug, having MIC values of 0.125 µg/ml.

In strain T0 grown without drug (figure 10B), one clone (UUA1) achieved a MIC value of 1 µg/ml. All the other clones remained with lowest MIC values of 0.125 µg/ml. All 18 clones were susceptible to fluconazole. In the same T0 strain grown with fluconazole (figure 10C),

the MIC values are overall higher compared to the same T0 strain grown without drug. Three different populations (UUA5,UCU4 and UCU5) achieved the highest MIC value (MIC~ 256 µg/ml), therefore achieving resistance to the drug.

In strain T1 (figure 11B) grown without drug, two clones achieved MIC values of 0.5 µg/ml and 0.25 µg/ml. All the other clones remained with 0.125 µg/ml. In T1 strain grown with fluconazole (figure 11C), MIC values are overall higher compared to the same T1 strain grown without drug, as in strain T0. Four clones achieved resistance: intermediate MIC~64 µg/ml in UUA3, MIC~128 µg/ml in CUG6 and UCU5 and MIC~256 µg/ml in UUA5.

In strain T2 grown without drug (figure 12B), one clone (UUA2) achieved a MIC value of 0.5 µg/ml, and other three clones (UUA3, UCU2, UCU3) achieved MIC values of 0.25 µg/ml. In T2 strain grown with fluconazole (figure 12C), the MIC values are overall higher. In this strain, 6 clones (UUA3, UUA4, UUA5, CUG4, CUG5 and UCU6) achieved resistance, UUA3 achieved 64 µg/ml while the other 5 clones reached 256 µg/ml.

Comparing the three strains between them, we can see that in strains T1 and T2 there is a higher number of clones achieving higher MIC values in the presence of drug. Thus, mistranslation alters the frequency of acquisition of fluconazole resistance during evolution.

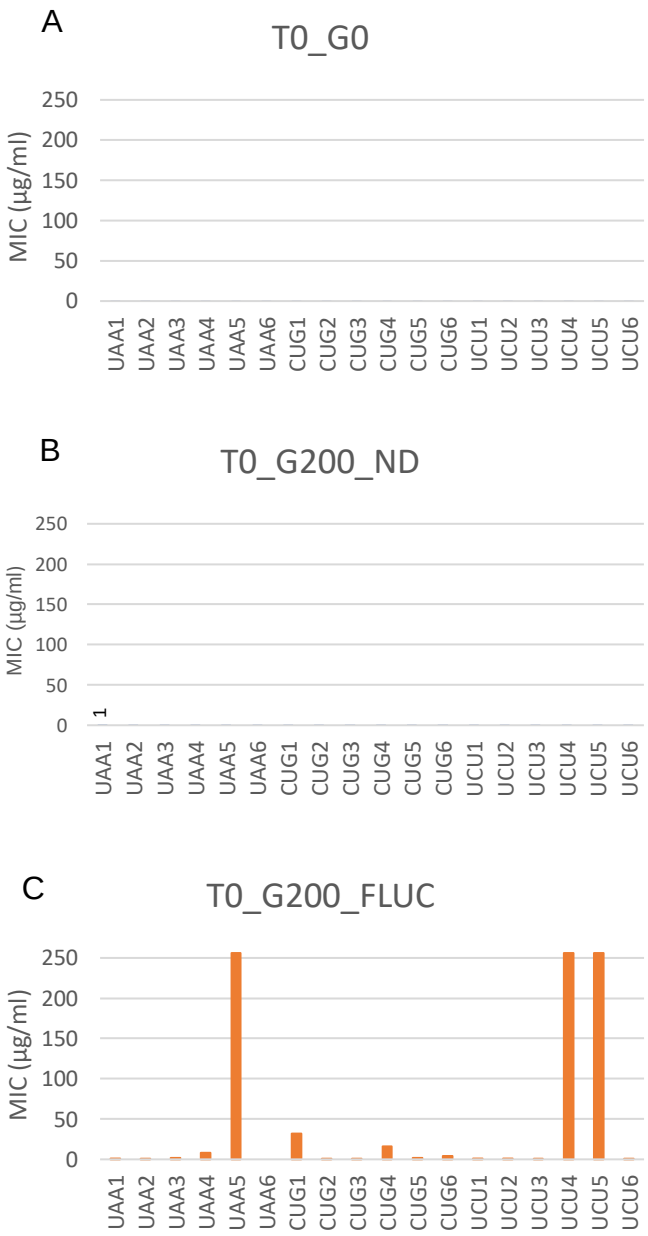

Figure 10- MIC values of strain T0 in the beginning (G0) **(A)** and end (G200) of the experimental evolution. ND means evolution with no drug **(B)** while FLUC means evolution in the presence of fluconazole **(C)**.

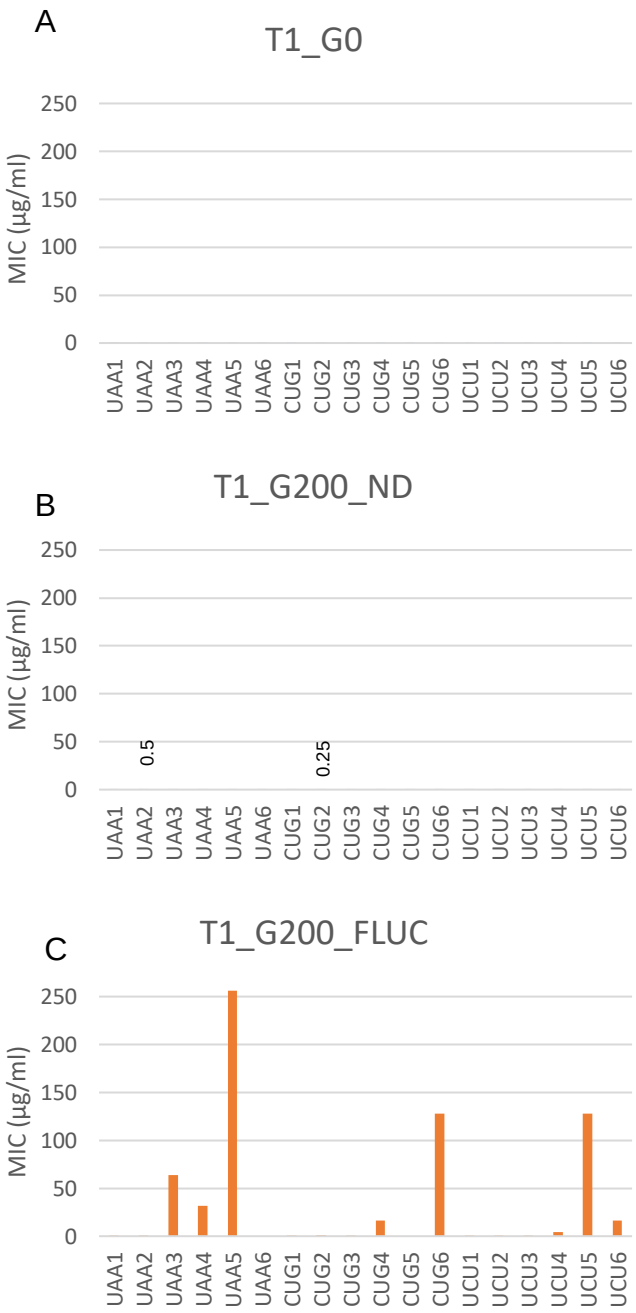

Figure 11- MIC values of strain T1 in the beginning (G0) **(A)** and end (G200) of the experimental evolution. ND means evolution with no drug **(B)** while FLUC means evolution in the presence of fluconazole **(C)**.

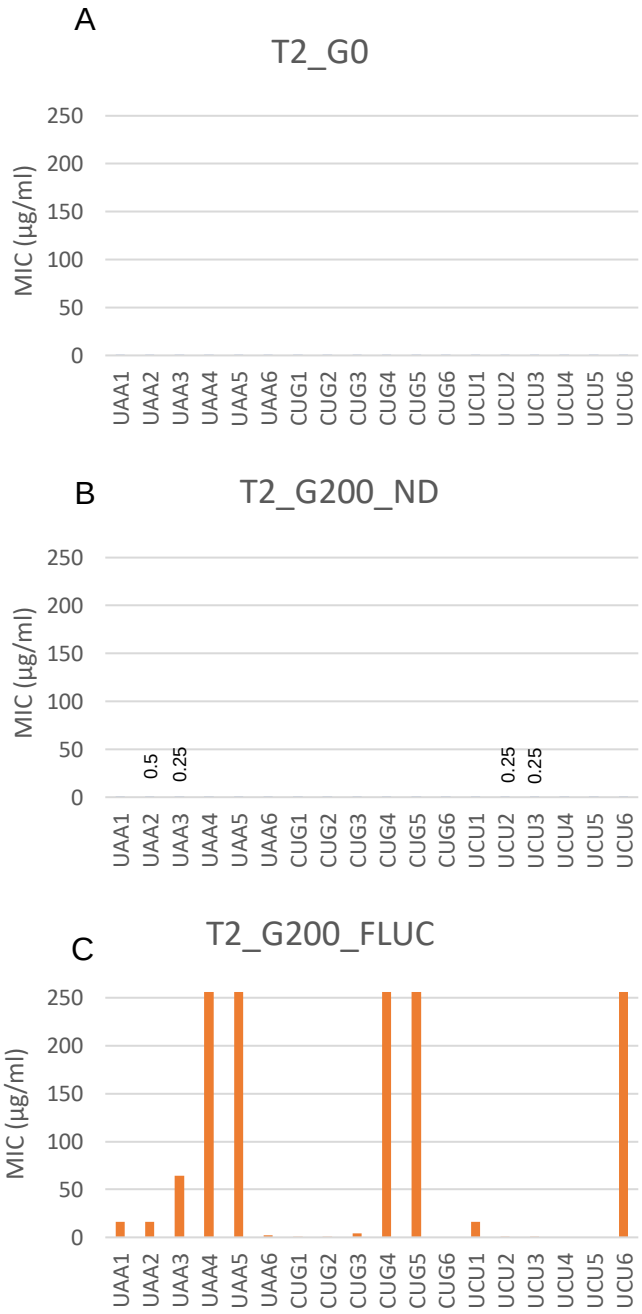

Figure 12- MIC values of strain T2 in the beginning (G0) **(A)** and end (G200) of the experimental evolution. ND means evolution with no drug **(B)** while FLUC means evolution in the presence of fluconazole **(C)**.

C.albicans cells have the ability to switch between different morphologies: yeast, pseudohyphae and hyphae according to the environment that they are exposed to [36][37]. Hyphal cells are associated with a more pathogenic state, whereas the yeast form is associated with dissemination in the bloodstream. To evaluate the effect of mistranslation

and the presence of fluconazole on the morphology of cells, *C.albicans* cells were observed under the microscope, to detect differences in morphology between T0, T1 and T2 strains.

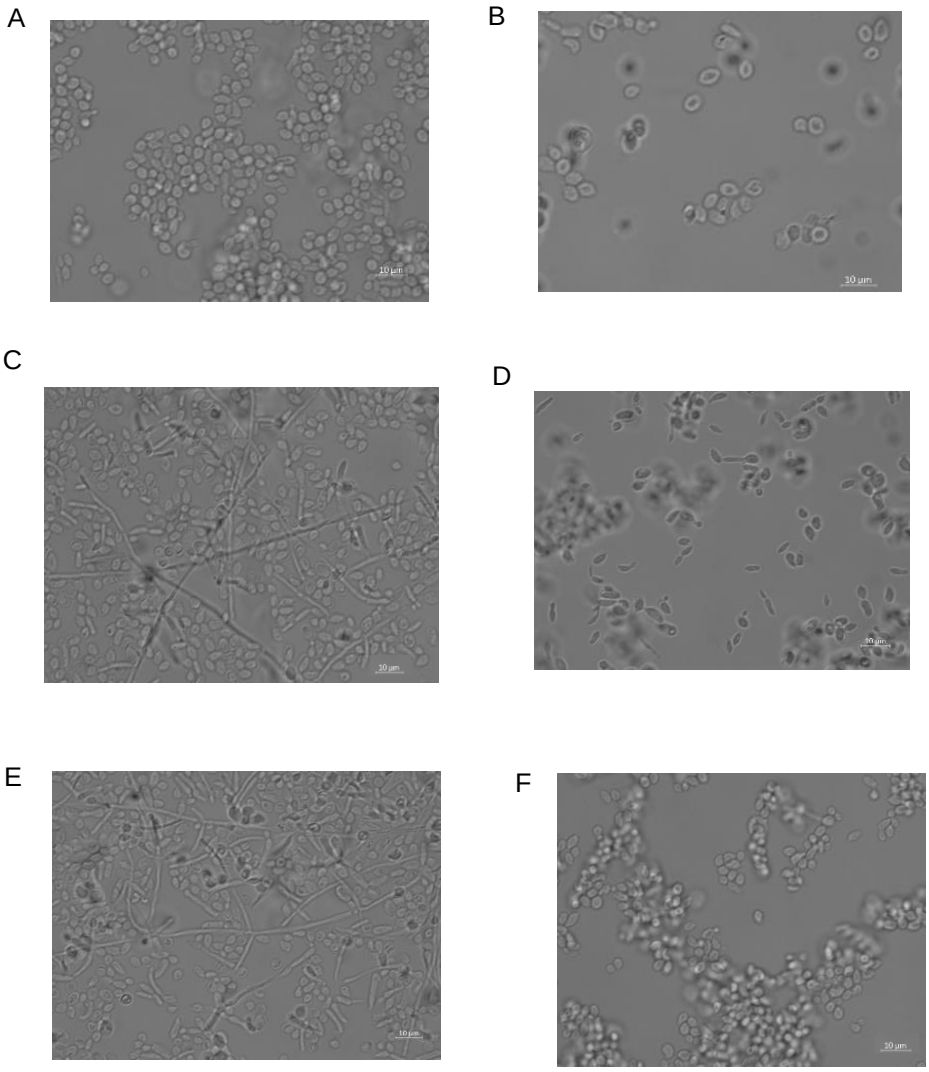

Figure 13- *C.albicans* cells observed under the Zeiss microscope. **(A)** T0 strain grown without drug **(B)** T0 strain grown with drug. **(C)** T1 strain grown without drug **(D)** T1 strain grown with drug **(E)** T2 strain grown without drug **(F)** T2 strain grown with drug. The scale bar represents 10 µm.

According to the results in figure 13, it's visible a difference in the morphology of the *C.albicans* cells between the different strains (T0, T1 and T2) and conditions (growth with and without drug). In T0 strain (figures 13A and 13B) cells have the normal ovoid yeast shape typical of *C.albicans* cells in the proliferative state. In strains T1 and T2 grown without drug (figures 13C and 13E respectively) it's observable the presence of hyphae, particularly in the strain T2. In strain T1 grown with drug (figure 13D), cells aren't as ovoid, and have a

more elongated shape compared to the T0 strain. Also, in strain T2 grown with fluconazole (figure 13F) *C.albicans* cells are organized in clusters of cells. The presence of clusters of cells is a common adaptation mechanism of *C.albicans* cells to stress, in this case, the higher ambiguity of T1 and T2 strains and the presence of fluconazole.

2. Measure mistranslation levels during the evolution of drug resistance

To see if throughout the experimental evolution there were changes in mistranslation levels of strains T0, T1 and T2, a gain-of-function reporter system based on the yeast enhanced green fluorescent protein (yEGFP) was used (described in section 2.5 of materials and methods). This reporter system allows the quantification of mistranslation through measurement of fluorescence intensity by flow cytometry.

Among the 18 clones used, 6 clones functioned as positive controls (containing the UUA-Leu codon at position 201 of the yEGFP gene), 6 functioned as negative controls (containing the UCU-Ser codon at position 201) and the other 6 were reporter clones (containing the ambiguous CUG codon at position 201).

All 6 clones were grown overnight at 37ºC in liquid medium until an OD_{600nm} of 0.8-2.0, and aliquots were analysed using the flow cytometer BD Accuri C6 after being filtered. Due to the presence of clusters of cells in strain T2 (as shown in figure 13F), we can't exclude the fact that a fraction of the population is not considered in the fluorescence analysis. Figures 14A, 14B and 14C show examples of GFP fluorescence intensity of T0 non-evolved positive control (UUA_{201} codon), T0 non-evolved negative control (UCU_{201} codon) and T0 non-evolved reporter clone (CUG_{201} codon) respectively. Figure 14D show GFP fluoresce intensity of a T2 reporter (CUG_{201} codon) non- evolved. We can see that the profile of the T2 reporter non-evolved (figure 14D) is different compared to the T0 non-evolved reporter clone (figure 14C).

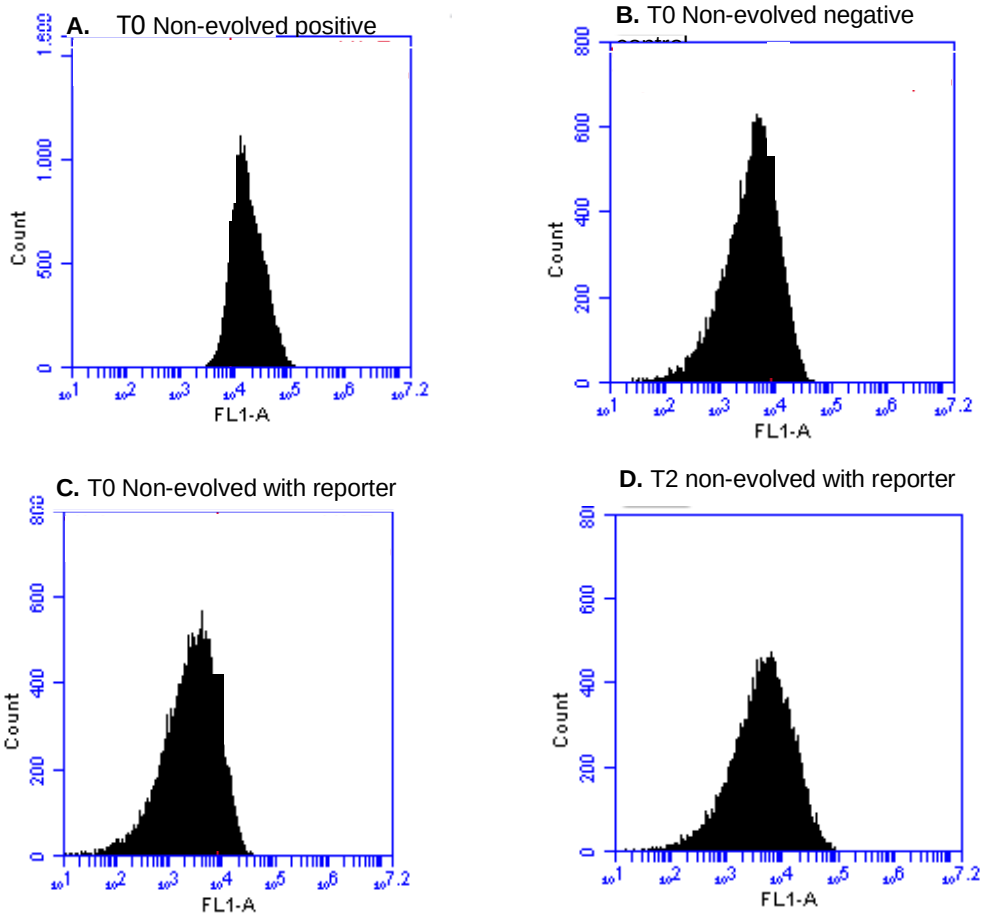

Figure 14- Example of GFP fluorescence intensity profiles (FL1-A) of T0 and T2 cells carrying the mistranslation reporter. **(A).** Non-evolved T0 positive control (UUA_{201} codon) **(B).** Non-evolved T0 negative control (UCU_{201} codon) **(C).** Non-evolved T0 containing the reporter (CUG_{201} codon) **(D).** Non-evolved T2 containing the reporter (CUG_{201} codon).

After normalization of fluorescence intensity levels, values of mistranslation were calculated.

Figure 15 shows the percentage of mistranslation throughout the experimental evolution in the different strains. The percentage of mistranslation in strain T0 remains similar throughout the experimental evolution (figure 13A). In the beginning of the evolution the ambiguity levels were about 3% and this value was maintained in passage 10 (200 generation) , both with and without drug.

In strain T1, results show an increase in mistranslation rate between passage 0 and 10 (figure 15B). In the beginning of the experimental evolution, mistranslation rate is about

14%, increasing to 34% in the end of the evolution with no drug. In passage 10 with fluconazole mistranslation levels are approximately 24%.

In strain T2, there is a very pronounce decrease of mistranslation rate between the passages 0 and 10 (figure 15C). In the beginning of the experimental evolution the ambiguity level was about 71%, decreasing to about 36% in passage 10 with and without drug. This decrease was explained in a previous master's thesis developed in the host laboratory. This work showed a deletion of the second copy of the tRNACAGLeu gene inserted at the RPS10 locus, rendering a strain similar to T1 (Mónica Barbosa master's thesis).

Overall, we can say that in all strains, mistranslation rate in passage 10 with and without fluconazole was similar. On the other side, the three strains showed different trends throughout the experimental evolution, strain T0 maintained the mistranslation rate, while strain T1 increased and strain T2 decreased mistranslation levels.

It's important to notice that no correlation was observed between changes in mistranslation levels and alterations in MIC values.

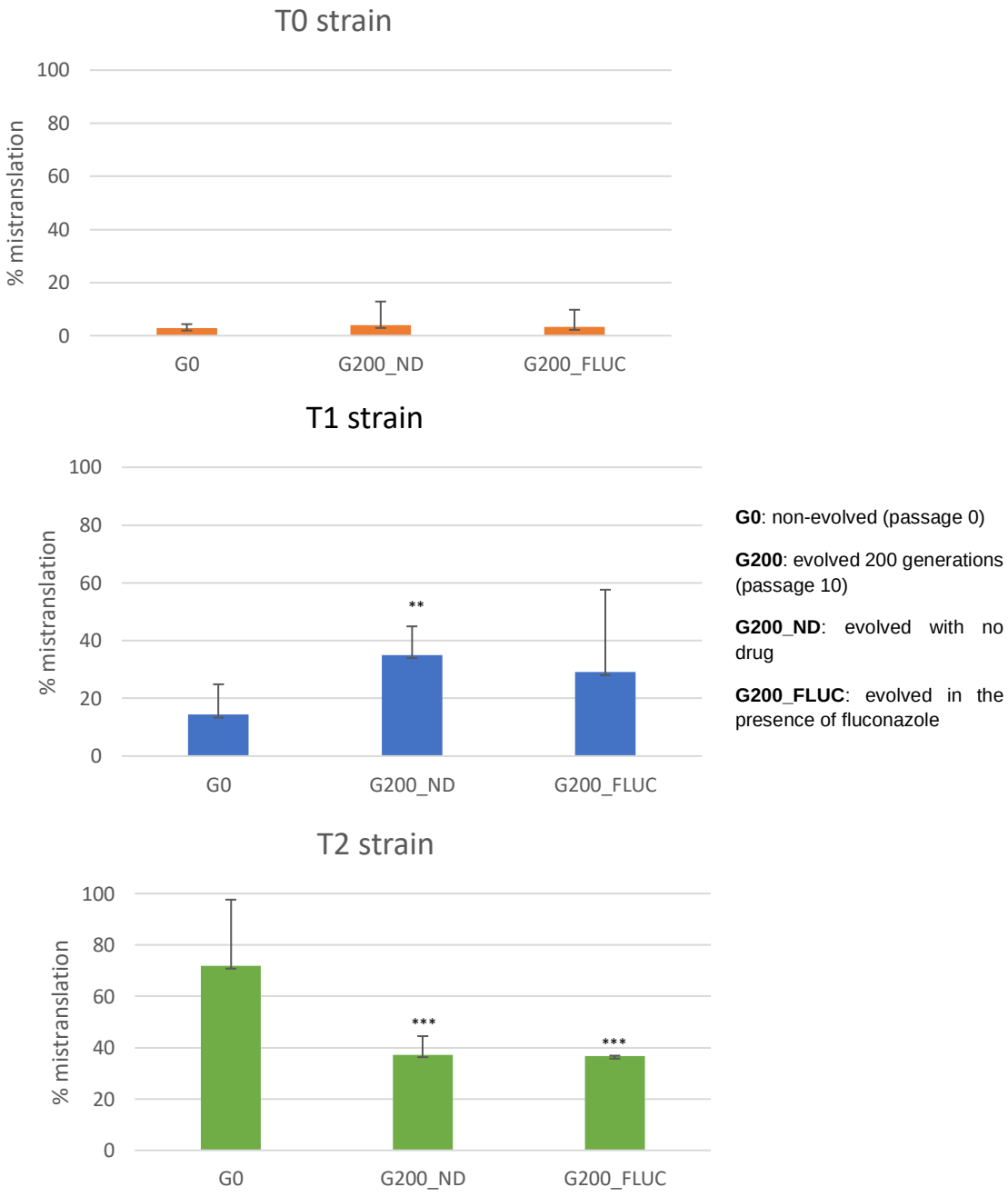

Figure 15- Percentage of mistranslation throughout the experimental evolution in strains T0, T1 and T2. **(A)** Percentage of mistranslation in strain T0. The values range from 2,93±1,41 in passage 0; 3,89±8,96 in passage 10 without fluconazole and 3,20±6,56 in passage 10 with fluconazole. **(B)** Percentage of mistranslation in strain T1. The values range from 14,34±10,52 in passage 0; 34,95±9,98 in passage 10 without fluconazole and 24,28±28,58 in passage 10 with fluconazole. **(C)** Percentage of mistranslation in strain T2. The values range from 71,75±25,86 in passage 0; 37,929±7,18 in passage 10 without fluconazole and 36,273±0,14 in passage 10 with fluconazole. Data represent the mean ± standard deviation of duplicates of 18 independent clones (***p<0.001; **p<0.01; *p<0.1 student's t-test relative to the non-evolved cells).

3. Genomic alterations induced by mistranslation and fluconazole

3.1. Ploidy Analysis

C. albicans is mostly found as a diploid organism, but when exposed to environmental stresses, it can undergo genomic changes as an adaptation mechanism to the new conditions. These genomic changes range from SNPs, indels and LOH events (Loss of Heterozigozity) to chromosome duplication, amplification and deletion. These events can induce changes in the ploidy level, and other forms of *C.albicans* can be found [67].

To see the effects of the experimental evolution, with and without fluconazole, on the ploidy level, flow cytometry experiment was performed. All eighteen clones from the strains T0, T1 and T2 were used, from initial and final passages (P0 and P10 respectively), grown with and without fluconazole. Additionally, strain SN148 was added as a 1N control. After growing overnight at 37ºC, all clones were treated with propidium iodide to stain the cells before analysis of DNA content by flow cytometry.

Fluorescence intensity of G1 and G2 peaks of the cell cycle (figure 16) were defined for each individual sample, and for the SN148 control. The first peak corresponds to the G1 phase, and the second peak to the G2 phase. The ploidy level of the samples is obtained by determining the mean G1 fluorescence of each sample relative to the mean G1 fluorescence of the control strain with known ploidy, in this case the SN148 diploid sample. When DNA content of the sample is in the range 1,8-2,2N, the sample is considered a "normal" diploid sample, meaning that there are no big variations in the sample's genome compared to the control sample (such as aneuploidies).

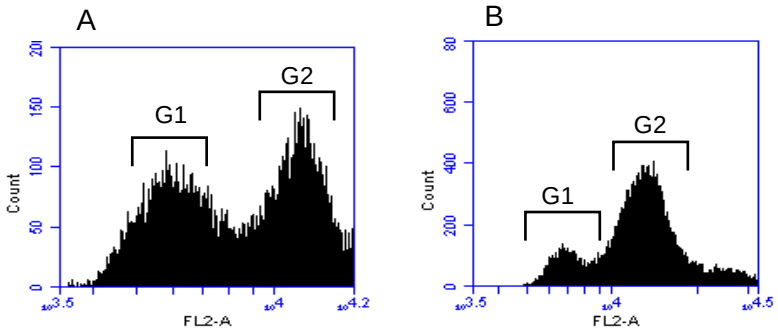

Figure 16- PI fluorescence intensity (FL2-A). **(A)** SN148 control strain **(B)** clone T1_ UCU5, grown with fluconazole. The first peak corresponds the the G1 phase of the cell cycle, and the second peak to the G2 phase. G1/G2 ratio of control and test samples must be 2 to confirm correct gating. From the mean fluorescence intensity values of these two peaks, the ploidy level of the samples is obtained. G2/G1 ratio of control and test samples must be 2 to confirm correct gating.

Figure 16 shows an example of PI fluorescence intensities, in one SN148 control sample and a clone from passage 10 evolved with drug. Figures 17, 18 and 19 show ploidy levels of T0, T1 and T2 clones throughout the experimental evolution.

In T0 strain, clones from the passage 0 (figure 17A) and passage 10 grown without drug (figure 17B) showed a ploidy level that fit in the 1.8-2.2N range and are therefore considered diploid. However, in passage 10 with fluconazole (figure 17C), 3 clones had 3N, 2.4N and 2.8N values; and therefore it's very likely that these clones have aneuploidies.

In strain T1 (figure 18), all clones show a ploidy level that fit in the 1.8-2.2 range, being diploid samples.

In T2 strain, in passage 0 (figure 19A) all clones were 2N. However, in passage 10 grown without fluconazole (figure 19B), only one clone had a higher ploidy value (~2.26), and therefore that clone can be aneuploid. Major differences were detected in T2 clones evolved with fluconazole (fig 19C), since 12 of the 18 clones appear to be aneuploid. In the majority of these clones, the values were higher than 2.2, ranging from 2.26 to 2.98; only one clone showed a value lower than 1.8. It is noteworthy that clones T2_UUA3, T2_UUA4, T1_UUA5, T2_CUG4, AND T2_UCU6 are also clones that acquired resistance during evolution, meaning that 5 out of 6 resistant clones are aneuploid.

Overall, all clones of all strains at the beginning of the experimental evolution are diploid. Only in passage 10 clones started to change ploidy particularly in passage 10 evolved with drug. When comparing the three strains, the majority of the clones with different ploidy values were from T2 strain, which is the higher mistranslating strain. Thus, the experimental evolution in the presence of drug seems to influence the DNA content of *C.albicans* clones.

C.albicans is known for its genome instability, and the occurrence of aneuploid chromosomes is well documented, including loss or gain of an entire chromosome or a large portion of chromosome [68]. The values of ploidy found outside the 1.8-2.2 range can be attributable to gross chromosome rearrangements, like the loss or gain of a big portion of a chromosome. We can't pinpoint the exact type of aneuploidy that occurred and further studies need to be performed to elucidate this topic.

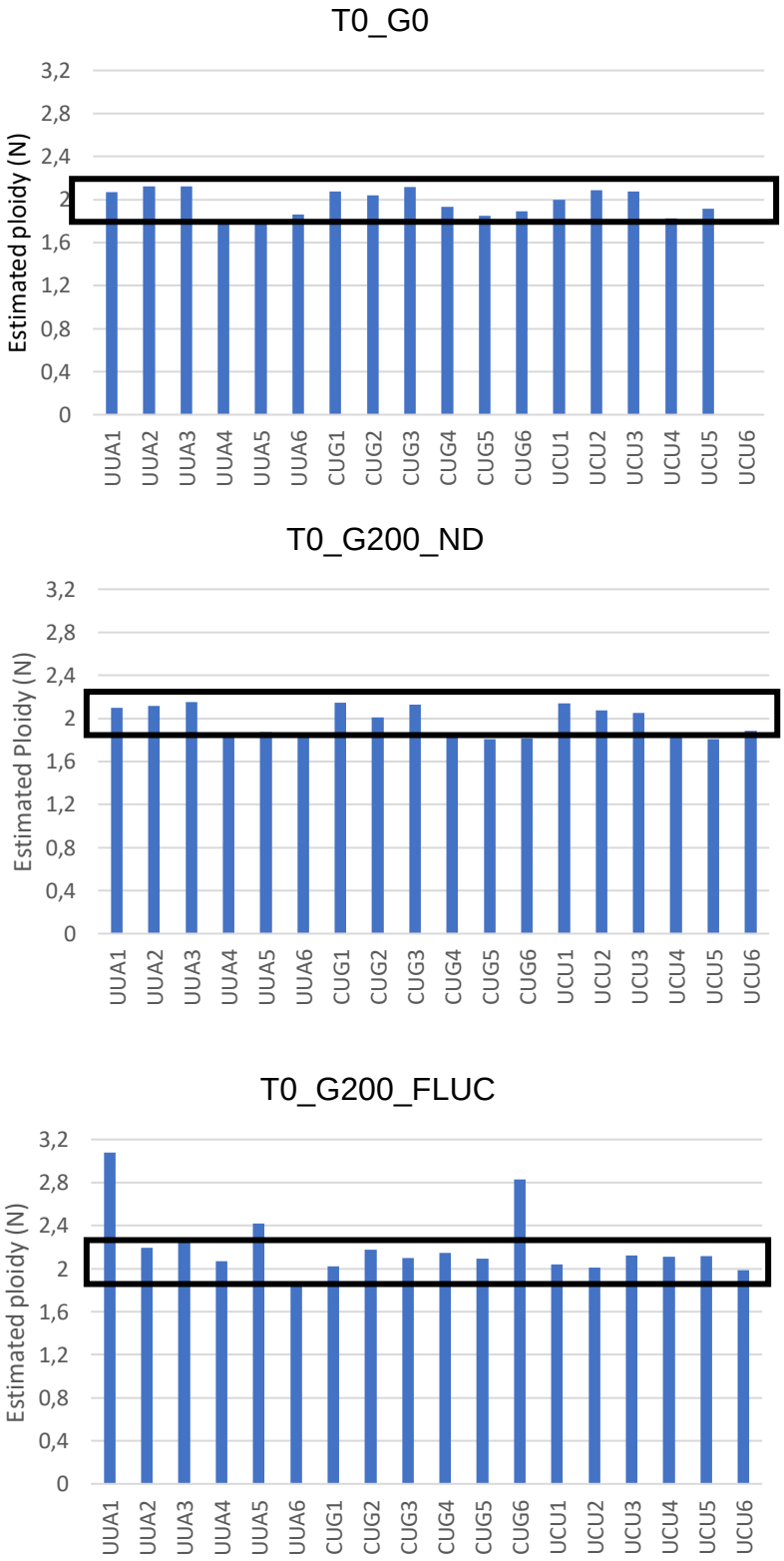

Figure 17 -Ploidy of T0 strain throughout the experimental evolution **(A)** Passage 0 **(B)** Passage 10 grown without fluconazole **(C)** Passage 10 grown with fluconazole.

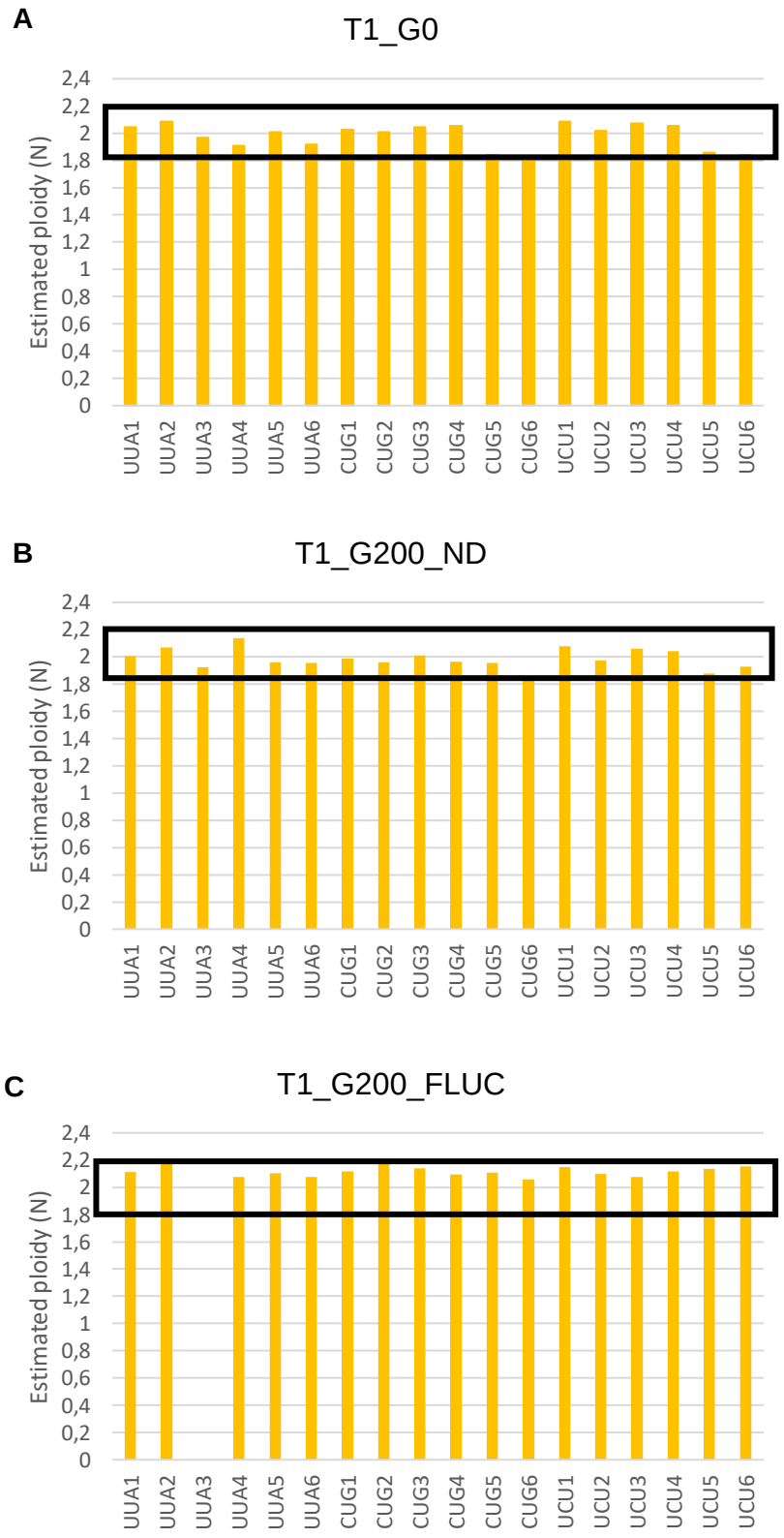

Figure 18 -Ploidy of T1 strain throughout the experimental evolution **(A)** Passage 0 **(B)** Passage 10 grown without fluconazole **(C)** Passage 10 grown with fluconazole.

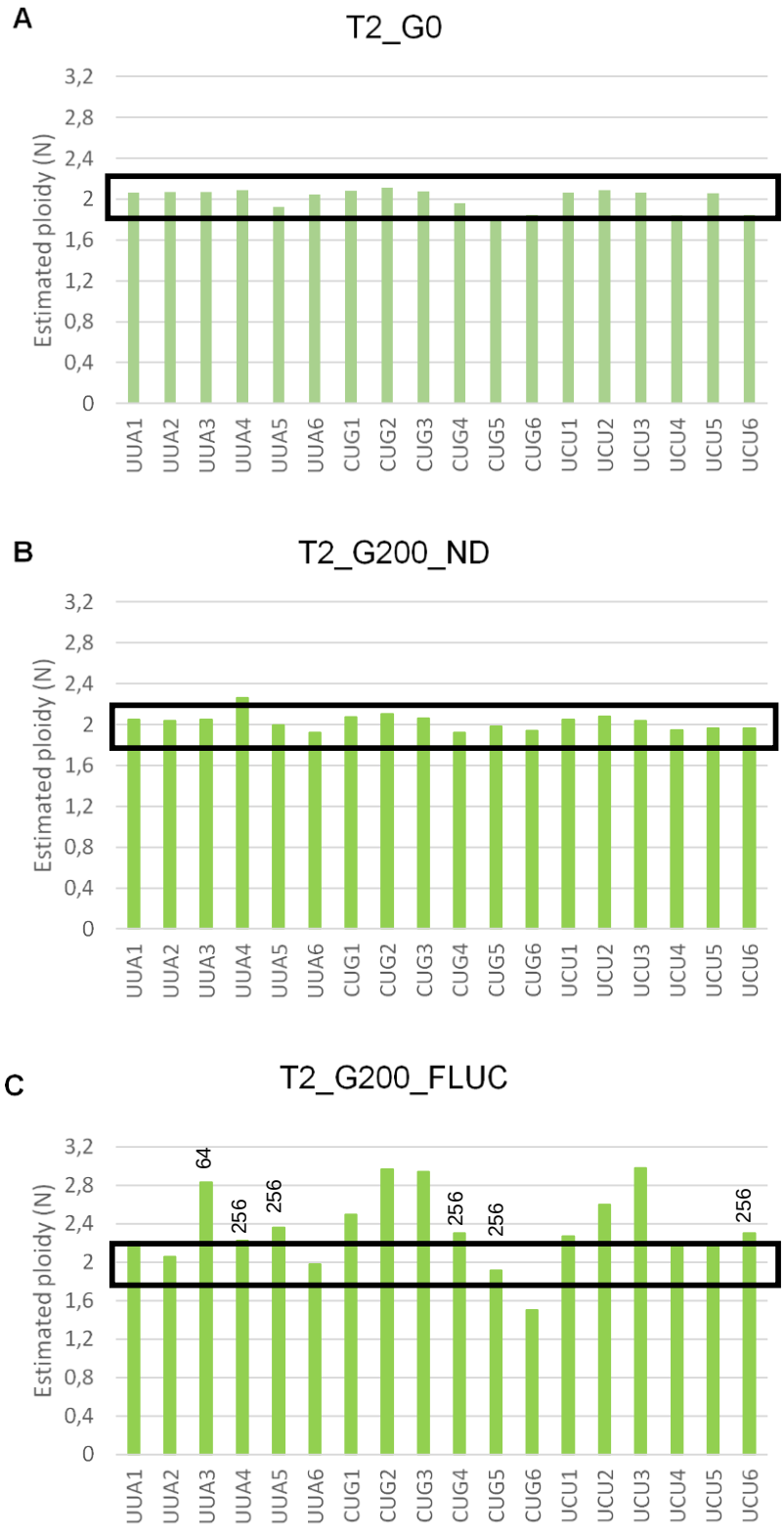

Figure 19 -Ploidy of T2 strain throughout the experimental evolution **(A)** Passage 0 **(B)** Passage 10 grown without fluconazole **(C)** Passage 10 grown with fluconazole. MIC values of resistant clones are shown at the top of the corresponding bar.

3.2. Genome re-sequencing

To gather more insight into the mechanism of acquisition of drug resistance by hypermistranslating strains, we performed a pilot experiment. Illumina genomic DNA libraries were generated from DNA isolated from three different *C.albicans* clones, with the goal of identifying possible genomic alterations associated with the acquisition of resistance to fluconazole. The three clones used were: non-evolved clone T1-UUA5 with low MIC (MIC~0.125 µg/ml); clone T1-UUA5 from the passage 10 evolved without drug, with low MIC (MIC~0.125 µg/ml) and one resistant clone T1-UUA5 from passage 10 evolved with drug (MIC~256 µg/ml) (table 3).

Table 3- *C.albicans* clones analysed by sequencing.

Name	Strain-Clone	Passage	Evolution	MIC (µg/ml)
T1_non-evolv	T1-UUA5	0	--	0.125
T1_evolv_ND	T1-UUA5	10	Without drug	0.125
T1_evolv_FLUC	T1-UUA5	10	With drug	256

The goal is to identify genomic alterations that may be associated with the acquisition of resistance to fluconazole. We can do so by comparing the genome of resistant clone evolved in the presence of fluconazole with a control clone from the beginning of the experiment. To do that comparison, we have to first identify the alterations that emerge in a clone evolved without drug, and then exclude those from the alterations in the resistant clone (Figure 20). Thus, mutations present in both resistant and non-resistant were defined as non-causal polymorphisms for antifungal resistance development.

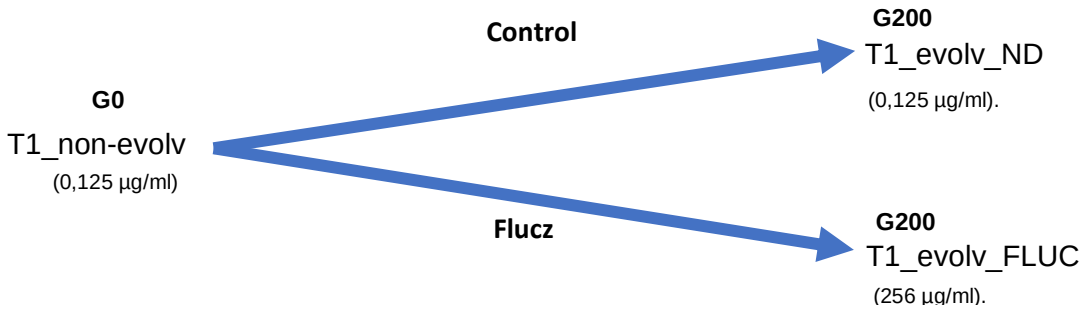

Figure 20- Scheme of the clones sequenced. ”Control” refers to the experimental evolution without fluconazole; “Flucz” refers to the experimental evolution with fluconazole.

Reads obtained from the three samples were mapped against the *C.albicans* reference genome, using the *C.albicans* SC5314 strain as the reference (www.candidagenome.org). Following quality filtering, the reads yield a coverage 191,24 in sample T1_evolv_ND, 115,38 in sample T1_evolv_FLUC and 131,11 in sample T1_non-evolv, with ~99% of the reads mapped for all samples (Table 4). Coverage by chromosome for each sample is shown in annex A.

Table 4- Read mapping statistics, number of reads, percentage of mapped reads and genome coverage of mapping.

	Genome coverage	**#Reads**	**%mapped reads**
T1_non-evolv	131,11	13204827	99,3
T1_evolv_ND	191,24	19338883	99,3
T1_evolv_FLUC	115,38	11675272	98,9

After mapping the reads, variants to the reference genome were determined, namely SNPs (Single nucleotide polymorphisms) and INDELs (Small insertion and deletion). Other types of genome alterations, such as loss of heterozygosity (LOH), copy number variation (CNVs) are still being analysed and are not included in this thesis.

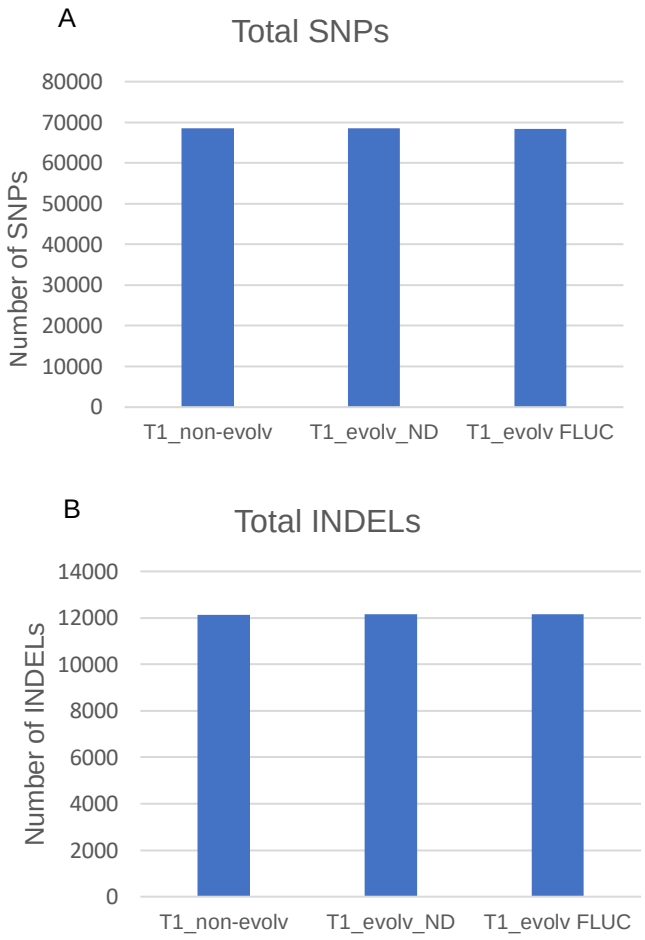

Figure 21- Total number of SNPs **(A)** and INDELs **(B)**.

Figure 21 shows the total number of SNPs and INDELs identified in all samples and their distribution per chromosome is in annexes B and C. By analysing the number of SNPs obtained (figure 21A), we can see that this number didn't vary much between the three samples, all having about 68.000 SNPs, which are distributed per chromosome in the same way. These values are consistent with the estimated >48.000 SNPs in strain SC5413 [69]. When analysing the number of INDELs obtained (figure 21B), once again the three samples are uniform between each other, having about 12.000 INDELs, distributed in the same way per chromosome.

As mentioned previously, the goal was to identify the genetic variants (SNPs and INDELs) that were specific for the resistant strain, T1_evovl_FLUC, as these are the ones that arise from the evolution with drug. Thus, the number of SNPs and INDELs that are unique in each sample was obtained (figure 22).

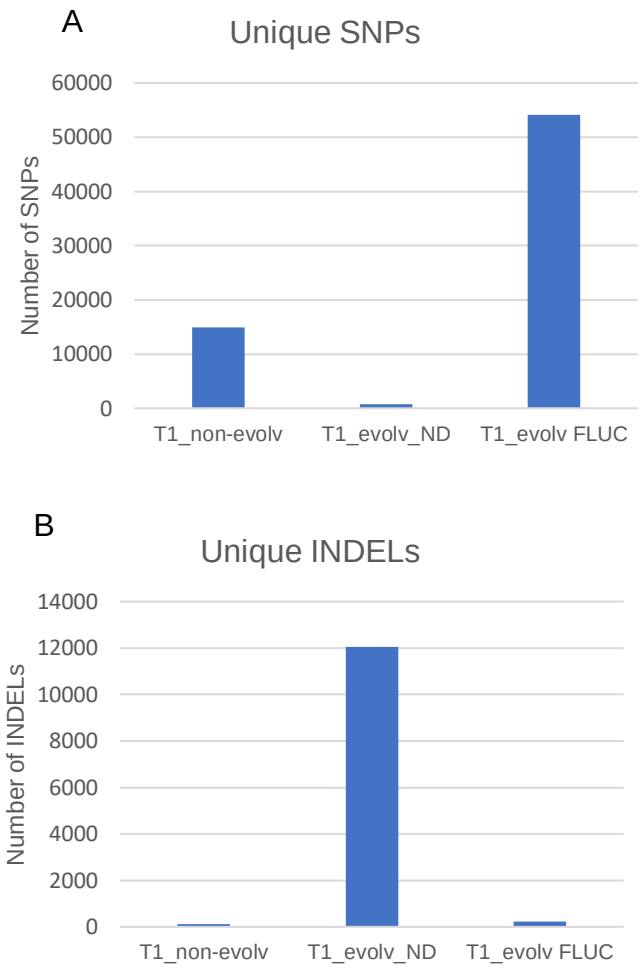

Figure 22- Number of unique SNPs **(A)** and INDELs **(B)** in each sample.

When analysing the results in figure 22A, we can see that the number of specific SNPs was different between samples. As was expected, the resistant clone has the highest number of specific SNP's, about 54.000, a value close to the 68.000 total SNPs found initially. Surprisingly, the sample evolved without drug had much fewer SNPs than the non-evolved sample.

Relatively to INDELs (figure 22B), the clone evolved without drug with low MIC had a much higher number of specific INDELs than the other samples. The clone from the beginning of the experiment showed the lowest number of specific INDELs, as was expected.

From the list of specific SNPs obtained we focused on the distribution of type of variants of the specific SNPs for each sample (figure 23). It's noticeable that, in all samples, the

majority of SNPs found are intergenic, about ~50%. As these are located in regions between genes, these mutations may or may not have an effect in the expression of the gene, and consequently on the protein produced. For example, if mutations are located in promoter region of the corresponding gene. The percentage of genes with missense SNPs is quite high for the resistant clone, about 40%, while only 19% and 28% for the other samples.

Figure 24 shows the distribution of type of variants of the specific INDELs for each sample. In the three samples, the majority of the INDEL's found are intergenic, about 70%. In sample evolved without drug, 65% of INDELs are intergenic, 5% are a conservative inframe deletion, 6% are a conservative inframe insertion, 7% are frameshift, and 17% have other types of variants. In the resistant clone, 70% of INDELs are intergenic, 3% are a conservative inframe deletion, 5% are a disruptive inframe insertion, and 22% are frameshift. In the clone from the beginning of the evolution experiment, 68% of INDELs are intergenic, 5% are a conservative inframe insertion, 5% are a disruptive inframe deletion, 9% are frameshift, and 13% have other types of variants.

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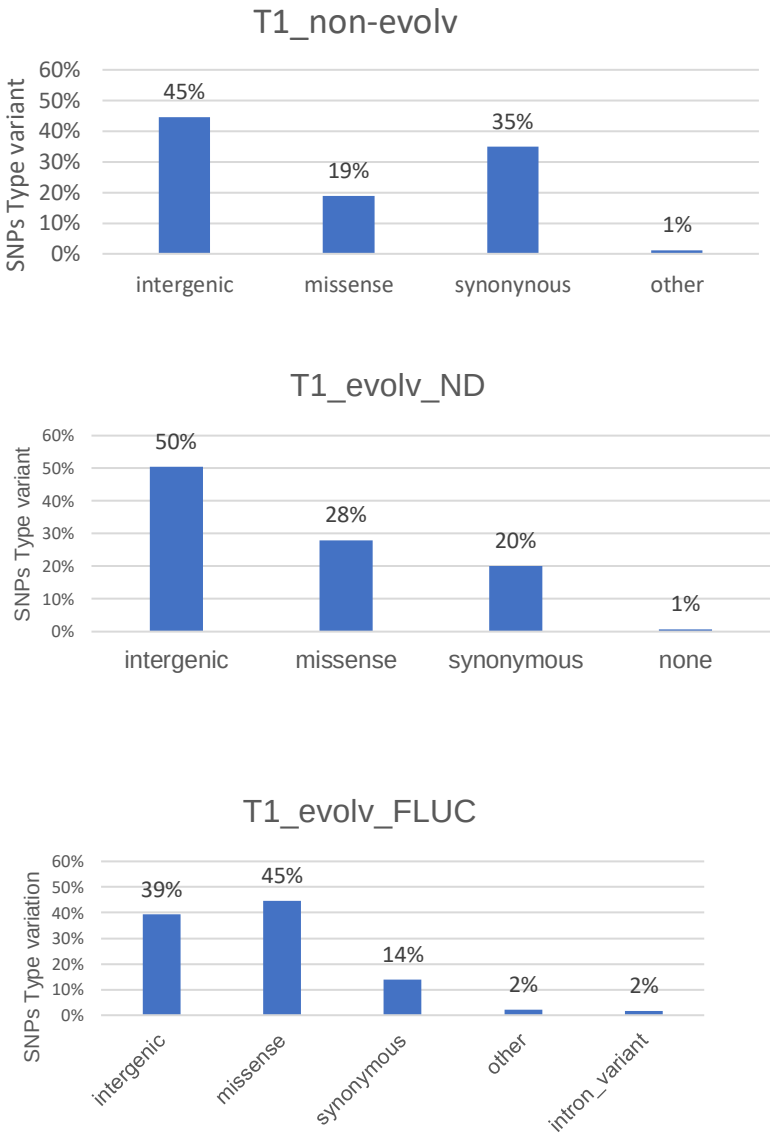

Figure 23- Distribution of type of variants of the specific SNPs for each sample.

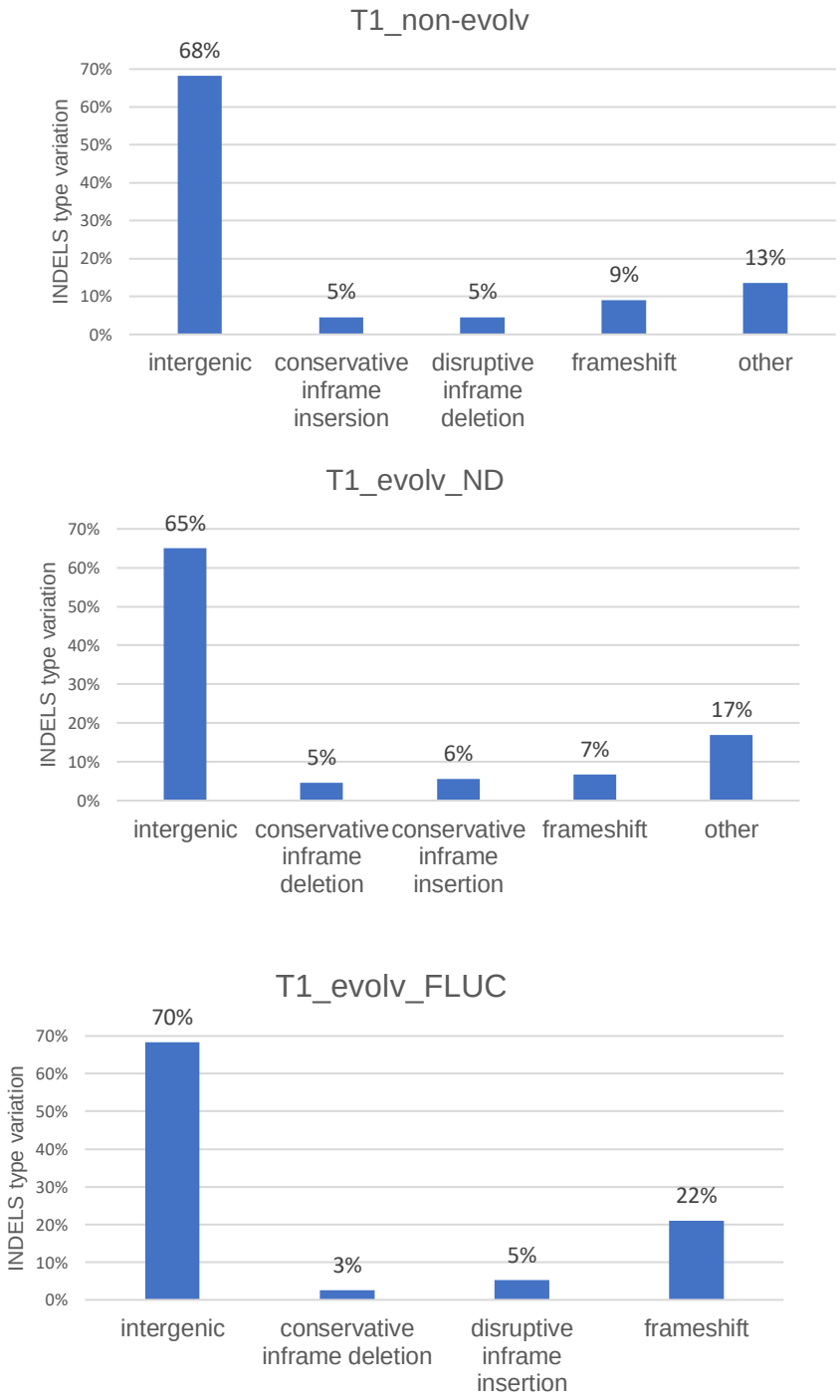

Figure 24- Distribution of type of variants of the specific INDELs for each sample.

In order to categorize genes with unique missense SNPs in the sample that acquired resistance, a functional enrichment analysis was carried out using the Gene Ontology (GO) Slim Mapper tool of Candida genome database. This process allowed the categorization of the genes by biological processes and functions associated with these genes, allowing us to understand if any of these alterations can be associated with the acquisition of fluconazole resistance.

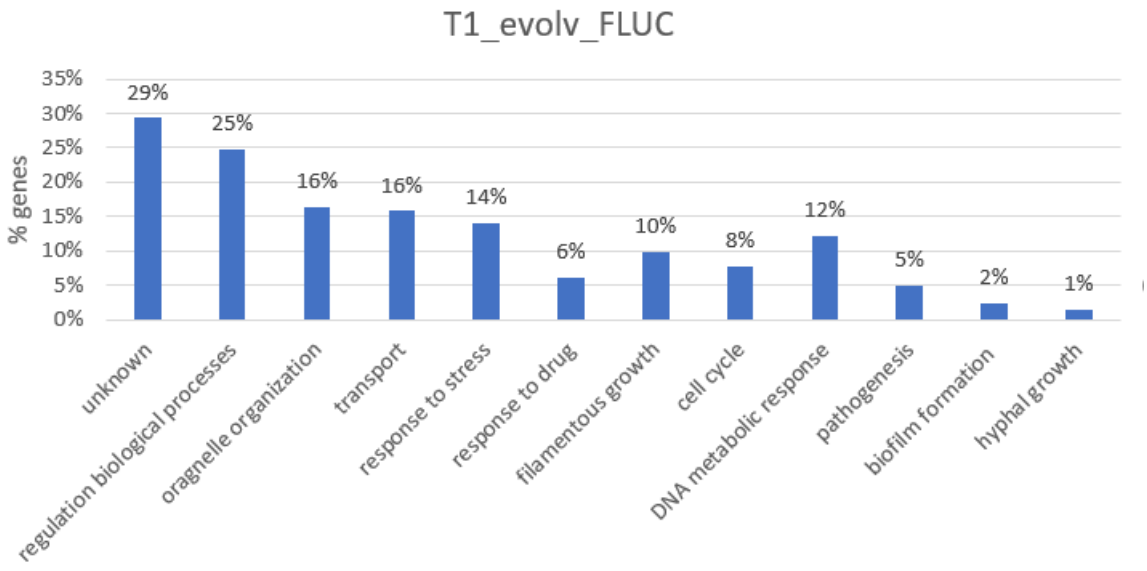

Figure 25- Distribution of genes with missense SNPs for the resistant clone (T1_evolv_FLUC; MIC~256µg/ml).

In the resistant clone (figure 25), the total number genes found with missense SNPs was 2620. Of these, 29,3% have an unknown function. 24,8% are involved in the regulation of biological processes, 16.4% are involved in organelle organization, and 15,8% involved in transport. Moreover, 14% of the affected genes are related to the response to stress, from which 6.2% (163 genes) are specifically involved in response to drug, 9.8% involved in filamentous growth, 7.8% involved in cell cycle, 5,6% involved in DNA metabolic processes, 5% involved in pathogenesis, 2.4% related to biofilm formation, and 1.4% involved in hyphal growth .

From 163 genes involved in response to drug, some are not described as involved in fluconazole resistance. According to the results obtained through functional enrichment analysis in the Candida Genome Database, eleven genes were found whose mutation

confers hypersensitivity to a toxic ergosterol analogue (DAG7, TAF4, TPO5, VID27, APL2, ARP8, BOI2, C2_09860C, C4_01090C, CAR2, FMO1, FMP45). Two genes (C5_03940C and CAP1) are involved in multidrug resistance, being C5_03940C a putative multidrug resistance protein upregulated by Efg1p, and CAP1 a transcription factor involved in apoptotic oxidative stress/response and multidrug resistance. CAS4 was found to be involved in azole sensitivity and hyphal growth, along with SOG2, which is also involved in azole sensitivity. Only 5 genes (DAG7, ERG11, ERG2, RAD50 and ROA1) were already described in the literature as possibly involved in azole resistance (table 5) .

Regarding samples T1_non-evolv and T1_evolv_ND, only 576 and 129 genes contained SNPs. Since the number is low, no functional enrichment was possible.

Table 5- Genes with SNPs associated with a drug response described in the literature.

Genes	Type	Chr	Nucleotide substitution	Aminoacid substitution	Drug response
DAG7	Protein coding	Chr 4	c.280G>C	p.Glu94Gln	hypersensitivity to toxic ergosterol analogues [70]
ERG11	Protein coding	Chr5	c.383A>C	p.Lys128Thr	hypersensitivity to multiple drugs [71]
ERG2	Protein coding	Chr1	c.143T>C	p.Leu48Pro	ergosterol biosynthesis gene associated with drug resistance [72]
RAD50	Protein coding	Chr3	c.1399T>C	p.Tyr467His	involved in response to oxidative stress and drug resistance [73]
ROA1	Protein coding	Chr1	c.979C>A	p.His327Asn	PDR-subfamily ABC transporter involved in sensitivity to azoles [74]

Relatively to the INDELs analysis, in the resistant clone, from the 38 genes with INDELs, 27 were unknown in the candida genome database. From the 11 genes left, 4 had a unknown biological function, 3 genes (FGR28, FGR51, FGR6-4) were involved in filamentous growth and response to stress, 2 genes (C1_14560C_A, C4_03230C_A) were involved in DNA metabolic response, 1 gene (ALS7) was involved in interspecies interaction, biofilm formation and cell adhesion. One gene (FMO1) was found to be involved in response to chemical and more specifically response to drugs.

From these results, it's noteworthy that there weren't found any genes with INDELs associated with response to drug in the resistant clone. This result was expected, as in the literature INDELs haven't been described as a mechanism of developing drug resistance to azole drugs.

4 Discussion

The emergence of *C.albicans* infections led to the widespread use of antifungal drugs. The most commonly used antifungal drug is fluconazole, due to its high bioavailability, high water solubility and low affinity to plasma proteins. The prolonged use of fungistatic antifungal therapies (such as fluconazole) increases the incidence of acquired antifungal drug resistance, which is the ability of the pathogen to evolutionarily develop mechanisms that lower their susceptibility towards a drug. This process involves genomic alterations ranging from chromosomal rearrangements to point mutations. These mutations can affect drug resistance in different ways, directly interfering with binding of the drug to its target or inducing gene expression changes that reduce drug susceptibility [75][76].This topic is still poorly studied, and further insights are needed to understand how resistance towards antifungal drugs emerges.

Several mechanisms of resistance to fluconazole have been described. They can be divided in three main categories: alteration of drug target, upregulation of drug transporters and cellular stress responses. The most studied mechanism is mutations of the drug target ERG11 gene, which decreases affinity to the drug and prevents drug binding. It has also been reported overexpression of the drug target ERG11, which decreases the impact of the drug on the cell [48][47][55]. Other important mechanism of drug resistance is overexpression of drug transporters (efflux pumps),including multidrug resistance gene MDR1, which is controlled by the transcription factor MRR1, and Candida drug resistance genes 1 and 2 (CDR1 and CDR2). [76] Another possible mechanism of azole resistance is the homozygosis of TAC1, which is required for the upregulation of the ABC drug transporters CDR1 and CDR2. Finally, another resistance mechanism is the alteration in the sterol biosynthesis pathway, through the inactivation of the ERG3 gene, which in turns leads to the inactivation of the C5-sterol desaturase, resulting in the reduction of ergosterol and accumulation of other sterols [76][77]. Altogether, studies suggest that *C.albicans* acquires resistance to antifungal drugs through the mechanisms described on table 6.

Table 6- Genetic bases of resistance towards azoles drugs.

Gene	Mode of resistance	Description
ERG11	Overexpression of drug target	increased concentration of lanosterol 14α—demethylase
ERG11	Alteration of drug target (mutations)	decreased lanosterol 14α—demethylase binding affinity for the drug
ERG3	Inactivation of ERG3	Inactivation of C5 sterol desaturase leading to alterations in ergosterol synthetic pathway
TAC1	Loss of heterozygosity Aneuploidy	Upregulation of ABC transporter genes (CDR1 and CDR2)
MDR1	Overexpression	Upregulates the expression of the ABC transporter genes leading to active efflux of the drug
MRR1	Overexpression	Upregulates the expression of MDR1 leading to active efflux of the drug
CDR1	Overexpression	Upregulation of the ABC transporter genes leads to active efflux of the drug
CDR2	Overexpression	Upregulation of the ABC transporter genes leads to active efflux of the drug

A previous study by Bezerra *et al* showed that strains with more Leu misincorporation (strain T2 with 67.29%) grew better in media containing fluconazole and itraconazole, suggesting that CUG ambiguity is relevant to evolution of antifungal drug resistance. Through genome sequencing it was shown that increasing CUG ambiguity leads to a rapid genome evolution through mutation and loss of heterozygosity (LOH) relative to the T0 control strain. It was observed a higher rate of genotype changes in the hypermistranslating strain. Overall this study suggested that mistranslation is associated with an increased frequency of genomic changes that can interfere with the response to drug treatment of *C.albicans* [26].

In this study, we used experimental evolution to determine the ability of hypermistranslating *C. albicans* strains to acquire resistance to fluconazole. By comparing changes in a hypermistranslating strain with those in a WT strain subjected to fluconazole treatment, we

could detect contributions of mistranslation to drug resistance at the genome level. The aim of this study was to see if tRNA mistranslation played a role in the acquisition of drug resistance, through the experimental evolution with and without the presence of fluconazole.

Clones evolved without the drug kept the MIC values low throughout the evolution and are susceptible to the drug, as they have MIC <8 µg/mL (figures 10, 11 and 12). Some of the clones evolved with drug were able to achieve resistance (MIC >64 µg/ml). In T0 strain, 3 clones had MIC~256 µg/mL; in T1 strain 5 clones had MIC>64 µg/mL; and in T2 strain 6 clones had MIC~256 µg/mL. Thus, as higher mistranslating strains (T1 and T2) had more resistant clones than the control strain (T0), it's possible to conclude that mistranslation alters the frequency of acquisition of fluconazole resistance during evolution.

Regarding mistranslation levels throughout the experimental evolution, strain T0 maintained levels of ambiguity; T1 strain showed a small increase in the mistranslation rate; and T2 strain showed a very pronounced decrease in the mistranslation rate (figure 15). In both T1 and T2 strains, there is a stabilization of the mistranslation rate at about 35%. This occurred even in the evolution with and without drug, suggesting that the physiological range of CUG ambiguity tolerated by *C. albicans* cells must be within the 3-35% range. Indeed, previous studies showed a variation between 1.5% and 28% in ambiguity under environmental stress, with minimal decrease in growth rate [23]. Also, mistranslation levels were similar between susceptible and resistant clones.

In order to identify mechanisms of acquisition of fluconazole resistance by hypermistranslating strains, we performed DNA content analysis and a pilot genome sequencing experiment to compare with previous results detailed in table 6 When analysing the ploidy level of the three strains throughout the experimental evolution, for all strains, in the beginning of the experimental evolution all clones were diploid. Only on passage 10 clones with varying ploidy levels and most of them were evolved with fluconazole. Thus, the presence of the drug seems to exert a pressure to which the *C.albicans* cells have to adapt, probably through genomic changes, ultimately leading to ploidy changes. It's noticeable that most clones with ploidy values outside the 1.8-2.2N range were from the hypermistranslating T2 strain, 12 out of 16 clones. In this strain, we also found that 6 of those 12 aneuploid clones acquired resistance, which can be indicative of a link between aneuploidies and the acquisition of resistance. *In vitro* evolution experiments have previously provided evidence that fungi can undergo ploidy changes during adaptive evolution. Many relevant fungi undergo ploidy changes as an adaptation to specific environments, for example exposure to antifungal drugs, growth on alternative carbon

sources and high temperature [40][78]. It's been shown that growth in the presence of fluconazole often results in a variety of ploidies and aneuploid changes in *C.albicans*. As this drug alters membrane fluidity, it causes abnormal cytokinesis and cell cycle effects that lead to polyploid and aneuploid formation. One of these aneuploidies is the formation of isochromosome 5L (i(5L)), confers fluconazole resistance due to the amplification of two genes, ERG11 and TAC1. Due to the high fitness benefit, i(5L) accumulates rapidly in the population, and in many cases is maintained over ~330 generations in the presence of fluconazole [40][79]. So far, we couldn´t identify the type of aneuploidy that occurred in clones of the hypermistranslating strain T2 but these studies are underway.

Besides ploidy analysis, Illumina genomic DNA libraries were created from DNA isolated from susceptible and resistant hypermistranslating clones, allowing us to identify possible genomic alterations associated with the acquisition of resistance to fluconazole. In this study, we focused on the unique SNPs identified in the evolved resistant clone. This hypermistranslating resistant clone had a much higher number of specific SNPs than the other samples. From these, we focused on the genes with missense SNPs, as these result in an amino acid change in the protein produced. In the resistant clone, from a total of 2620 genes with missense SNPs, only 6% (163 genes) were involved in response to drug; the other genes were involved other processes such as the regulation of biological processes, organelle organization and transport. Of these genes involved in drug response, 11 were described as hypersensitive to toxic ergosterol analogues, 2 genes were involved in multidrug resistance and 2 genes were involved in azole sensitivity. Only 5 genes had been previously described in the literature as possibly involved in developing drug resistance (DAG7, ERG11, ERG2, RAD50, ROA1). All these genes are protein coding, and the nucleotide substitution gives rise to an amino acid substitution that can affect the protein's normal function. In this case, all these mutants are described in the literature as involved in the drug resistance mechanism.

In one study by Sorgo et al., it was shown that Dag7 levels were elevated in cultures grown with fluconazole and that a mutation in this gene confers hypersensitivity to toxic ergosterol analogues, playing therefore a protective role against the azole stress [70]. The ERG2 gene encodes the enzyme C-8 sterol isomerase, which converts fecosterol in episterol. It's been shown that upregulation of ERG2 could provide conditions optimal for CDR1p and CDR2p activity, and also could potentially compensate for a partial inhibition of lanosterol demethylase by an azole antifungal agent [72]. The RAD50 gene is involved in double strain break repair (DSBR), being required for both homologous recombination (HR) and

non homologous end joining (NEH). By studying the involvement of DSBR in genome stability and antifungal drug resistance acquisition in *C.albicans* it was shown that RAD50 mutants effective in HR have higher genome instability and show elevated frequency of drug resistance colonies [73]. ROA1 is an ABC transporter, that has been implicated in drug resistance, being similar in structure to CDR1 and CDR2. It was observed that homozygous deletion of ROA1 leads to an increased resistance to azoles [74]. One possible mechanism to developing resistance, reported several times in the literature, is through point mutations or SNPs in the ERG11 gene. This gene is the target of azole antifungal drugs and encodes for the enzyme lanosterol 14-α demethylase. SNPs in the ERG11 gene cause aminoacid substitutions and configuration changes of the protein encoded by the ERG11 gene resulting in decreased affinity of the enzyme to azole [71][80]. Having found SNPs in these genes, specific for the hypermistranslating resistant clone, it's likely that these alterations play a role in the acquisition of drug resistance.

Relatively to the identified INDELs, the same analysis was carried out. However, the resistant clone didn't show any INDELs associated with drug response, and therefore INDELs do not seem to cause the development of resistance in this clone.

By sequencing three hypermistranslating clones, one resistant clone evolved with drug, one clone non-resistant evolved without drug, and a non-evolved clone, it was possible to identify genes specific for the resistant clone, that are described in the literature as involved in drug resistance. A larger scale study, with sequencing of all the clones from the three strains would provide further insights in the study of the role of mistranslation in acquisition of drug resistance.

5 Conclusions and future perspectives

The unique translational system of *C.albicans* and the presence of antifungal drugs are pressures that induce genome instability, which constitutes a powerful mechanism to develop drug resistance. Our results suggest that mistranslation plays an important role in the acquisition of drug resistance. Strains with higher mistranslation levels acquired resistance with more frequency than control strains. Sequencing of the entire genomes of hypermistranslating clones allowed us to identify variants in genes that may possibly be involved in the acquisition of drug resistance, such as the DAG7, ERG11, ERG2, RAD50 and ROA1 genes. Taken together, our data suggest that mistranslation mediated more rapid evolution of fluconazole resistance via a range of mechanisms, including the classical effects on efflux and ergosterol biosynthesis. Many other mechanisms not referred here are likely to also play a role in developing resistance, such as aneuploidy and LOH events.

Our data shows that mistranslation accelerates the evolution of resistance to fluconazole in *C. albicans* but the molecular determinants of acquisition of drug resistance in hypermistranslators need to be further studied. A large scale study, involving gene expression profiling and sequencing of all clones would be important in order to associate the genomic variants with the acquisition of drug resistance. This type of study would provide mechanistic insights into the role of mistranslation in acquisition of drug resistance.

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Annexes

Annex A- Read mapping statistics, coverage by chromosome.

	Coverage by chromosome								
	ca22chr1A	**ca22chr2A**	**ca22chr3A**	**ca22chr4A**	**ca22chr5A**	**ca22chr6A**	**ca22chr7A**	**ca22chrM**	**ca22chrRA**
A (T1_UUA5 P10 ND 0.125)	157,9	170,6	190,7	189,8	197	204,6	210,8	3090,5	190,9
C (T1_UUA5 P10 FLU 256)	99,7	105,8	115,5	116,9	119	130,6	125,8	1151,6	114
D (T1_UUA5 P0 ND 0.125)	114,5	120,8	132,5	132,3	134,2	138	142	1374,3	131,2

Annex B- Total number of SNPs and their distribution per chromosome for samples A, C and D.

SNPs	**A-M_S4**	**C-M_S2**	**D-M_S1**
	T1_UUA5 P10 ND 0.125	**T1_UUA5 P10 FLU 256**	**T1_UUA5 P0 ND 0.125**
ca22chr1A	18436	18385	18407
ca22chr2A	5942	5967	6011
ca22chr3A	4528	4508	4526
ca22chr4A	10034	9995	10027
ca22chr5A	7479	7526	7497
ca22chr6A	9250	9205	9213
ca22chr7A	2239	2255	2313
ca22chrM	1	0	0
ca22chrRA	10567	10600	10609
Total	68476	68441	68603

Annex C- Total number of INDELs and their distribution per chromosome for samples A, C and D.

INDELs	**A-M_S4**	**C-M_S2**	**D-M_S1**
	T1_UUA5 P10 ND 0.125	**T1_UUA5 P10 FLU 256**	**T1_UUA5 P0 ND 0.125**
ca22chr1A	3209	3197	3216
ca22chr2A	1403	1405	1399
ca22chr3A	974	976	971
ca22chr4A	1670	1674	1671
ca22chr5A	1227	1239	1228
ca22chr6A	1443	1425	1421
ca22chr7A	361	364	365
ca22chrM	0	0	0
ca22chrRA	1875	1868	1866
Total	12162	12148	12137

Annex D- Number of SNPs specific for each sample, and their distribution per chromosome in samples A, C and D.

SNPs	A-M_S4	C-M_S2	D-M_S1
	T1_UUA5 P10 ND 0.125	T1_UUA5 P10 FLU 256	T1_UUA5 P0 ND 0.125
ca22chr1A	141	14747	3734
ca22chr2A	61	4876	1186
ca22chr3A	32	3637	904
ca22chr4A	86	8200	1920
ca22chr5A	121	5953	1503
ca22chr6A	130	6172	3139
ca22chr7A	49	1972	418
ca22chrM	1	0	0
ca22chrRA	71	8595	2119
Total	692	54152	14923

Annex E- Number of specific INDELs for each sample, and their distribution per chromosome in samples A, C and D.

INDELs	A-M_S4	C-M_S2	D-M_S1
	T1_UUA5 P10 ND 0.125	T1_UUA5 P10 FLU 256	T1_UUA5 P0 ND 0.125
ca22chr1A	3187	35	40
ca22chr2A	1391	29	12
ca22chr3A	960	19	12
ca22chr4A	1654	38	15
ca22chr5A	1218	27	5
ca22chr6A	1412	20	7
ca22chr7A	357	21	6
ca22chrM	0	0	0
ca22chrRA	1858	33	14
Total	12037	221	111