

**Master Thesis of Medicine**

**Mémoire de Maîtrise en médecine**

**Influence of *CgPDR1* hyperactivity on the expression  
of *EPA*-family adhesins in *Candida glabrata***

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## II. ABSTRACT

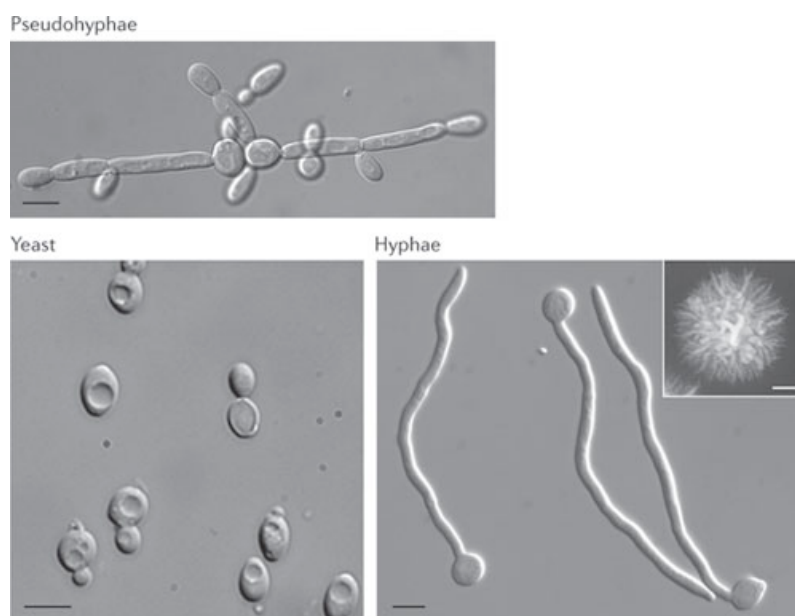
*Candida glabrata* is an emerging opportunistic pathogen that is known to develop resistance to azole drugs due to increased drug efflux. Resistance is mediated by a range of gain-of-function (GOF) mutations in *CgPDR1*, which are also known to enhance virulence. Recent data suggests that increased adherence to epithelial cells is partially responsible for the gained virulence, however it remains unclear which genes are implicated. In this study we used Real-Time quantitative PCR to analyze the influence of *CgPDR1* GOF mutations on the expression of several cell wall proteins that are either known or predicted to play a role in the adhesion of yeast to host epithelial cells. *CgPDR1* GOF mutations lead to an overexpression of different adhesins. While the adhesins were not regulated in a homogenous fashion for all the studied strains, we observed that *EPA1* played a prominent role being often up regulated as the only adhesin. An included non-subtelomerically located adhesin did not show any change in expression levels in GOF mutant strains compared to the wild type strain. Our findings point towards a complex regulation of adhesin expression through *CgPDR1*, implicating subtelomeric-silencing mechanisms. These processes could lead to enhanced adherence to host cells and thereby contribute to the observed gain in virulence in *CgPDR1* mutants.

**Keywords:** *Candida glabrata*, *CgPDR1*, *EPA*, Adhesin, qPCR

### III. INTRODUCTION

#### III.1. *Candida* species and candidiasis

*Candida* is a genus of yeasts that includes around 154 species. Certain species are frequent colonizers of human skin and mucous membranes and thus members of the normal flora of the skin, mouth, vagina, and stool. *Candida* species are also opportunistic pathogens, capable of causing infections following disruption of the normal flora, a breach in the mucocutaneous barrier or a defect in host cellular immunity. The microscopic features of *Candida* species show species-related variations. All species produce blastoconidia individually or in small clusters. Blastoconidia may be round or elongated. *C. glabrata*, for example, grows as a small, elliptical, unicellular budding yeast at all times. Rarely, buds of *C. glabrata* can adhere to one another in rudimentary short chains. In marked contrast, other *Candida* species like *C. albicans* form elliptical budding cells that are typically larger than those of *C. glabrata*; *C. albicans* also forms elaborate and well developed multicellular filaments or pseudohyphae which may be long, branched, or curved under certain growth conditions (Figure 1) (2). For most *Candida* spp. the morphology is fundamentally the same whether observed *in vitro* or *in vivo*.



**Figure 1**

**Morphology of *C. albicans*.** The figure shows the different morphological features of *C. albicans*. The inset in the hyphae panel shows the appearance of a hyphal colony that has been growing for 5 days on Spider medium. Scale bars in the main panels represent 5  $\mu\text{m}$ , and in the inset on the hyphae panel represents 1 mm. Reproduced from reference (2).

The most frequently isolated *Candida* species in human infections is *C. albicans*, the most abundant and significant species. However, *C. glabrata* and other *Candida* species are also isolated as causative agents of *Candida* infections. More importantly, there has been a recent increase in infections due to non-*albicans Candida* species. Currently *C. glabrata* accounts for up to 26% of bloodstream infections (BSIs) caused by *Candida* species in North America (6).

The *Candida* genus is currently the most common cause of opportunistic mycoses worldwide and the fourth most common cause of healthcare-associated bloodstream infections in the United States. In some hospitals it is even the most common cause (5). These infections tend to occur in the sickest patients. The clinical manifestations of *Candida* species infections range from local mucous membrane infections to widespread dissemination with multisystem organ failure and often a devastating outcome for the patient. Major established risk factors for *Candida* species infection include the following amongst others (7):

- Broad-spectrum antibiotics and colonization by *Candida* species
- Decreased host defense (post transplantation immunosuppression, AIDS, neutropenia, genetic immune deficiency conditions)
- Surgery, particularly major abdominal surgery
- A prolonged stay in the ICU
- Central venous catheters

The prevalence of different *Candida* species varies considerably amongst the different hospital services. *C. glabrata* strains are the most common *Candida* species isolated from solid organ transplant (38.4% of infections) and hematopoietic stem cell transplant recipients (32.7% of infections) (5), two groups of patients receiving frequent azole antifungal prophylaxis. On the other hand, *C. glabrata* infections are almost absent in neonatal intensive care units where use of antifungal prophylaxis is generally low (Figure 2).

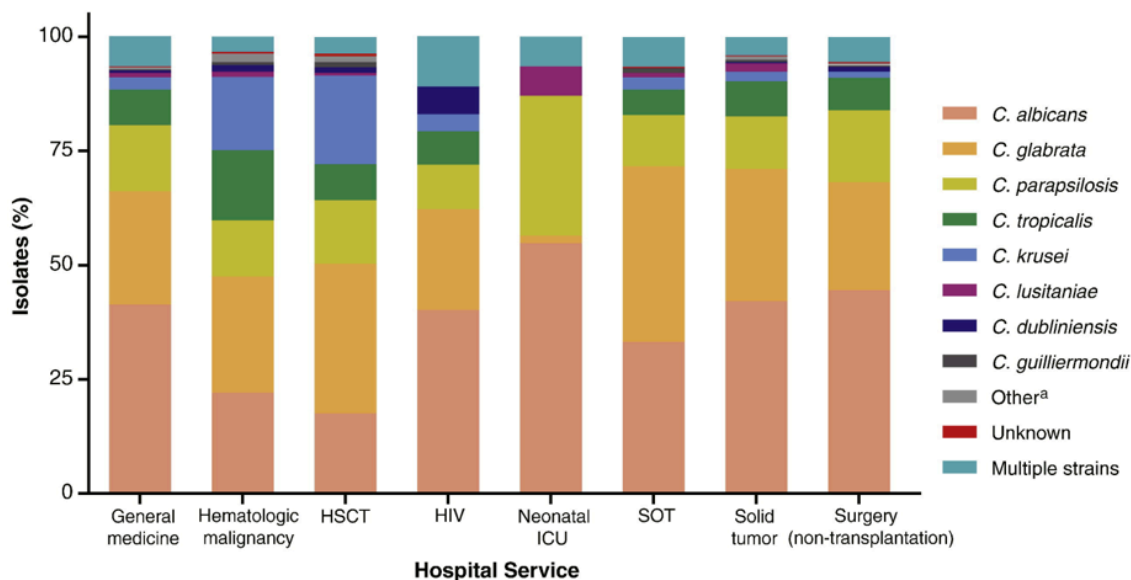


Figure 2

**Allocation of different *Candida* species amongst patients with candidemia in different hospital services.** <sup>a</sup>Includes *C. utilis*, *C. fermentati*, *C. kefyr*, *C. rugosa*, *C. famata*, *C. fennica*, *C. lambica*, and *C. lipolytica*. HSCT = hematopoietic stem cell transplantation; ICU = Intensive care units; SOT = solid organ transplant. Reproduced from reference (5).

### III.2. *Candida glabrata*

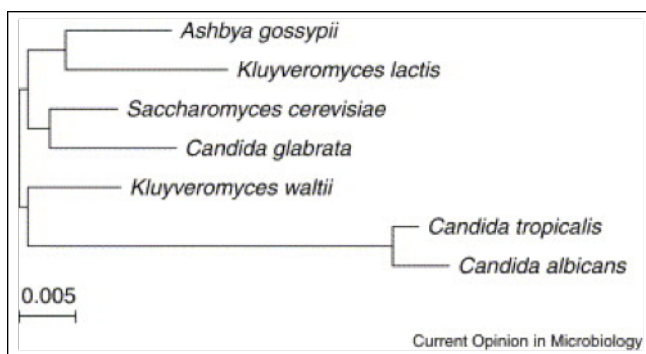
*C. glabrata*, a haploid, asexual member of the Ascomycetes once believed to be non-pathogenic, is currently regarded as the second most prevalent yeast pathogen in humans (6). In the environment *C. glabrata*, like *C. albicans*, is exclusively found in association with mammals, reflecting the success of the commensal lifestyle of these yeasts. Both species cause a similar constellation of BSIs and mucosal infections and have a primarily clonal population structure (Table 1). But despite these similarities *C. glabrata* and *C. albicans* are phylogenetically quite distinct (Fig. 3), suggesting that *C. glabrata*'s ability to infect humans emerged independently from that of other *Candida* species. *C. glabrata* shares common ancestry with several *Saccharomyces* species and, in spite of its name, clearly belongs to a clade different from that of other *Candida* species (1). Results of this evolutionary relatedness are, amongst others, a largely conserved chromosomal structure between *S. cerevisiae* (or baker's yeast) and *C. glabrata*, as most genes have orthologues in the other species, and a similar stress response (1). However, one question left unanswered is the difference in pathogenicity between *C. glabrata* and its non-pathogenic relative *S. cerevisiae*. Attributes like

biofilm formation, increased adherence to host cells and invasion into host tissue, stress resistance and a reduced susceptibility to antifungal drugs (Fig. 4) seem to play a crucial role in the acquisition of pathogenicity of *C. glabrata*.

**TABLE 1, Comparison of *C. glabrata*, *C. albicans* and *S. cerevisiae***

	<i>C. glabrata</i>	<i>C. albicans</i>	<i>S. cerevisiae</i>
Ploidy:	Haploid	Diploid	Diploid
Virulence:	Opportunistic pathogen	Opportunistic pathogen	Non-pathogenic
Major sites of infection:	Oral, vaginal, disseminated	Oral, vaginal, disseminated	Non-infectious
Mating genes:	Present	Present	Present
Sexual cycle:	Unknown	Known (cryptic)	Known
Clonal population structure:	Yes	Yes	No
Phenotypic switching:	Present	Present	Absent
True hyphae:	Absent	Present	Absent
Pseudohyphae:	Present	Present	Present
Biofilm formation:	Present	Present	Present
Major adhesins:	Lectins (encoded by EPA genes)	Lectins Hwp1 adhesin Als adhesins (ECM substrates)	Lectins (encoded by FLO genes) Sexual agglutinins
Auxotrophy:	Niacin, thiamine, pyridoxine	None	None
Azole resistance:	Innate resistance	Susceptible	Susceptible
Mitochondrial function:	Petite positive	Petite negative	Petite positive

Table adapted from reference (4)



**Figure 3**

**Phylogeny of *Candida* species and other hemiascomycetes.** *C. glabrata* and *S. cerevisiae* are phylogenetically closely related and are both quite distinct from other pathogenic *Candida* species. The evolution of the *S. cerevisiae* genome included a whole-genome duplication; this duplication occurred prior to the divergence of *S. cerevisiae* and *C. glabrata*. Reproduced from reference (4).



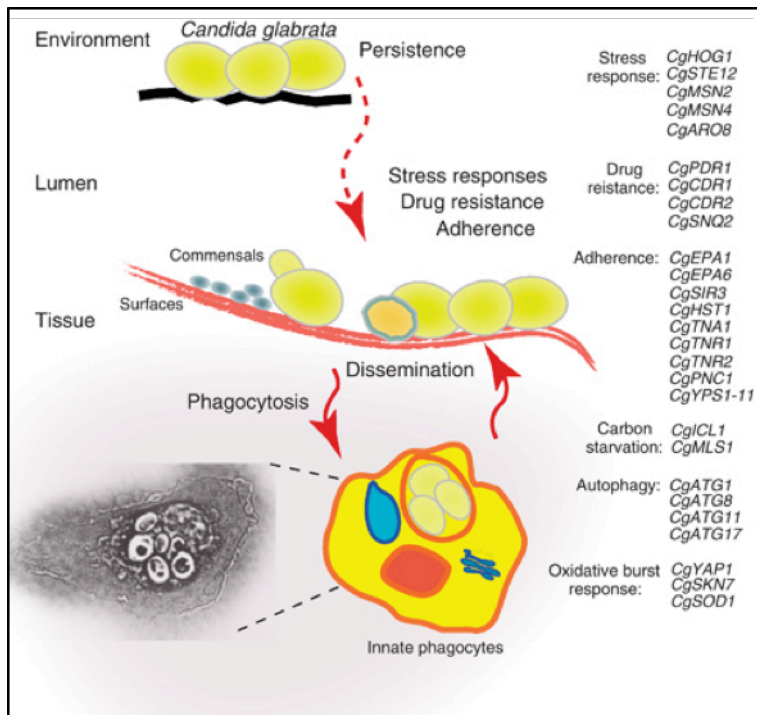


Figure 4

**Pathogenicity factors and dissemination mechanisms of *C. glabrata*.** *C. glabrata* cells can persist in environmental surfaces for several months. As a commensal, *C. glabrata* is found as part of the flora on interior mucosal areas of mammals such as the gut. In immunocompromised patients, cells are able to disseminate into tissues and cause organ failure. Patrolling phagocytes are responsible for the extinction of invading yeast pathogens. The right panel lists genes thought to be important for the pathogenicity of *C. glabrata*. Re-produced from reference (1).

### III.3. Treatment of *C. glabrata* infections and azole resistance

*C. glabrata* infections can be treated with several antifungal agents including amphotericin B, azoles and echinocandins. However *C. glabrata* has a relatively low drug susceptibility compared to other *Candida* species, particularly regarding azole drugs. Azole drugs are a class of antifungals that have been widely used for the treatment of candidiasis ever since the approval of fluconazole, the most prominent member of the azole family, by the United States Food and Drug Administration (FDA) in 1990. It is a first line treatment for many fungal infections including disseminated candidiasis in non-neutropenic patients or *Cryptococcosis* (8). Azole antifungals work primarily by inhibiting the cytochrome P450-dependent enzyme lanosterol 14-alpha-demethylase, encoded by the *ERG11* gene. This enzyme is necessary for the conversion of lanosterol to ergosterol, a vital component of the cellular membrane of fungi (4).

One of the reasons for the rise in *C. glabrata* infections in recent decades has been its high drug resistance, in particular towards different azole antifungals. It has been shown that this azole resistance is almost exclusively mediated by enhanced drug efflux which in turn is caused by overexpression of multidrug transporters of the ABC (ATP Binding Cassette) family, among which are at least *CgCDR1*, *CgCDR2* and *CgSNQ2* (9, 10).

### III.4. *C. glabrata CgPDR1*

A major regulator of these ABC drug efflux transporters in *C. glabrata* is *CgPDR1*, a zinc finger transcription factor, which combines functional attributes of transcription factors *PDR1* and *PDR3* from *S. cerevisiae* (11). Deletion of *CgPDR1* results in an increased susceptibility of *C. glabrata* to azole antifungals via the loss of overexpression of the involved ABC efflux transporters. In clinical isolates resistant to azole drugs, *CgPDR1* exhibits so-called gain of function (GOF) mutations, which are responsible for the overexpression of ABC drug efflux transporters (*CgCDR1*, *CgCDR2* and *CgSNQ2*). There is not only one specific mutation responsible for the azole resistance of the different clinical isolates, but a strikingly high diversity amongst the *CgPDR1* GOF mutations with as many as 67 described up to now (12, 13).

Another surprising feature of GOF mutations in *CgPDR1* is their role in fungal-host interaction. It has been shown that GOF mutations in *CgPDR1* are associated with enhanced virulence and fitness in murine models. Mice infected with *C. glabrata* strains containing these GOF mutations showed higher fungal organ burden and shorter survival time in a model of systemic infection, where neutropenic mice were challenged with tail vein injections of different *C. glabrata* strains (13). The enhanced virulence of azole resistant *PDR1* mutants in this study was a rather unexpected finding since it is generally accepted that the development of drug resistance in microbes is usually associated with a decline in virulence or fitness (14).

A possible mechanism behind the enhanced virulence of *C. glabrata* strains with *CgPDR1* hyperactivity could be a globally increased adherence to different epithelial cell lines and a decreased competitive uptake by macrophages (Fig. 5). Adherence to host epithelial surfaces is a key step in the host-pathogen interaction and is required for the successful establishment of colonization and infection by yeasts. A previous publication from our lab showed that hyperactive *CgPDR1* alleles cause an increased adherence of *C. glabrata* yeast to CHO-Lec2 cell monolayers in comparison to the wild-type allele in an isogenic strain background (15). This strongly suggests the involvement of adhesins in the enhanced virulence mediated by *CgPDR1* GOF mutations.

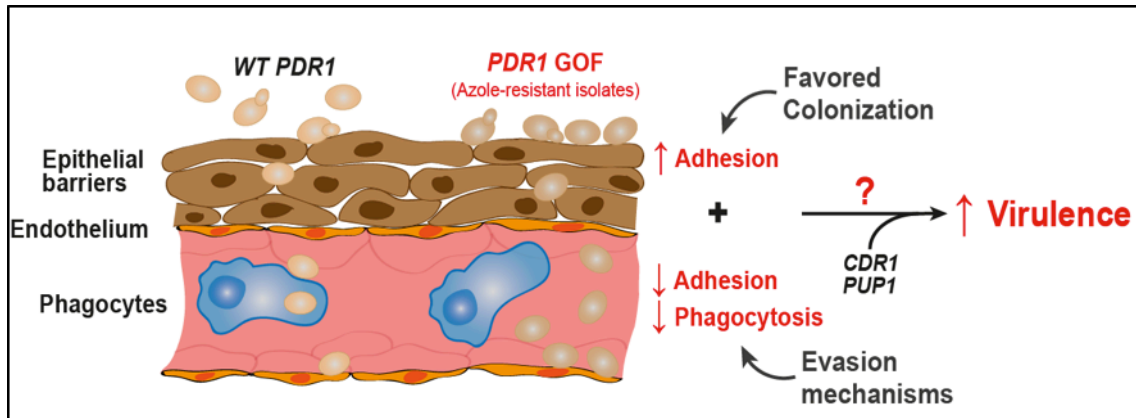


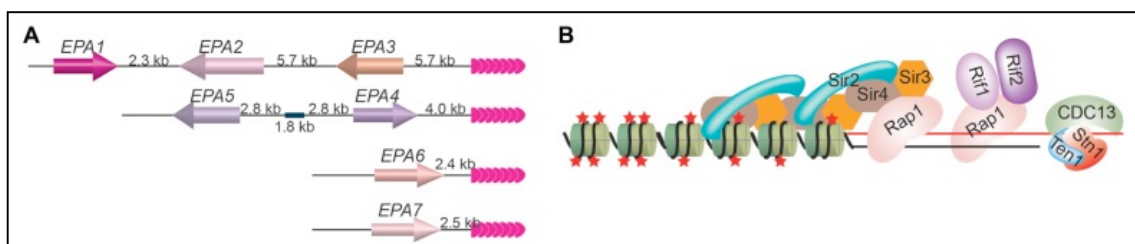
Figure 5

***CgPDR1*-mediated interactions of *C. glabrata* with host cells.** The figure illustrates the enhanced adherence of *CgPDR1* to epithelial cells, favoring colonization, and the decreased adherence to phagocytes, resulting in a decreased uptake and phagocytosis. These two mechanisms are possibly implicated in the enhanced virulence of *CgPDR1* GOF mutant strains.

### III.5. Adherence to host cells and Epa-adhesins

In fungi the cell wall has multiple roles, providing structural support to the cell but also forming the interface between the yeast and its environment. In pathogenic fungi, cell wall proteins (CWPs) mediate interactions with host cells, allow adherence and, in some cases, even invasion of the host. CWPs are therefore an important virulence factor in pathogenic fungi (16). While not the only one, an important family of cell wall anchored adhesins in *C. glabrata* are the Epa adhesins. This large sub-family of glycosylphosphatidylinositol (GPI) anchored CWPs is composed of 17 members in the reference *C. glabrata* strain CBS138. Earlier studies reported an even larger number of *EPA* genes, namely 23, in the BG2 strain, another widely studied *C. glabrata* strain (17, 18). For instance, orthologues of *EPA4* and *EPA5*, present in BG2, are lacking in the CBS138 strain. Furthermore, none of the orthologous *EPA* genes in the different strains present a perfect homology (17). The structure of Epa proteins is typical of adhesins: an effector domain is followed by a low complexity region that is often spiked with internal tandem repeats called “megatellites”. A C-terminal GPI anchor signal is required for adhesin function at the cell surface and therefore the sequences for the majority of the Epa proteins contain hydrophobic sequences for attachment of GPI anchors at their C-termini.

Remarkably, 44 of the 67 putative adhesins in *C. glabrata* and 14 of the 17 *EPA*-genes in CBS138 are encoded in subtelomeric loci (19) (Figure 6A). In this location they are subjected to chromatin-based transcriptional silencing. Silencing is initiated by Rap1 a duplex telomere DNA binding factor, which functions by recruiting a complex of proteins encoded by the *SIR2*, *SIR3* and *SIR4* genes. In current models, Sir2 possesses the key catalytic activity of the Sir complex. Sir2 is a NAD<sup>+</sup>-dependent histone deacetylase, which deacetylates histones H3 and H4 of a targeted nucleosome. This in turn is thought to provide high affinity binding sites for Sir3 and Sir4, which serve a primary structural role in the repressed chromatin. The Sir complex has the ability to move from the telomere itself across sub-telomeric regions sequentially deacetylating adjacent nucleosomes by Sir2 (4, 20, 21) (Figure 6B). Transcriptional silencing in *C. glabrata* has been shown to regulate transcription at four sub-telomeric *EPA* loci that encompass the coding regions of *EPA1–7*. Silencing depends, at least in part, upon the proximity of the silenced gene to the telomere, and decreases as the silenced gene is at longer distances from the telomere. Yeast strains carrying mutations in the genes implicated in chromatin based silencing mechanisms, such as a *sir3Δ* strain, are hyper adherent to a variety of cultured epithelial cell lines, owing to derepression of three *EPA* genes in particular: *EPA1*, *EPA6* and *EPA7* (22).



**Figure 6**

**Location of EPA1-7 and subtelomeric chromatin based silencing mechanisms.** (A) *EPA1–7* are located at subtelomeric loci in *C. glabrata*. The positions of seven *EPA* genes at their respective chromosome end loci are shown. Pink arrowheads illustrate telomere repeats. (B) Illustration of the chromatin-based silencing mechanism in budding yeast. Rap1 is the duplex telomere DNA binding factor, while Cdc13/Stn1/Ten1 binds to single-stranded telomere DNA. Rap1 recruits Sir3 and Sir4, which in turn recruits Sir2. Sir3 and Sir4 can also interact with histones directly. Sir2's deacetylase activity maintains the hypoacetylated state of histones and thereby decreases expression of a given gene. Red stars represent histone acetylation groups; green cylinders, nucleosomes. Figure reproduced from reference (3).

The currently available data points toward a strong implication of Epa adhesins in adherence to host cells and also virulence of *C. glabrata*. It has been shown that *epa1Δ* mutant strains display reduction to background levels in adherence to CHO Lec2 epithelial cells (23). A mutant strain, deficient of the three major adhesins known to date (*epa(1,6,7)Δ*), displayed a decreased capability to colonize the bladder in a murine model of urinary tract infection (21). Another study showed that a *C. glabrata* strain lacking the *EPAI-3* cluster exhibits a decreased virulence and produces a lower fungal burden in kidneys in a murine model of disseminated infection (18).

Interestingly, microarray data presented in a previous publication of our lab (24) shows that a cluster of known and putative adhesins, including *EPAI* and *EPAI2*, is regulated in the same manner by all the tested *CgPDR1* GOF mutations. This is remarkable, because different *CgPDR1* mutations show otherwise very different pictures of gene expression regulation, rarely controlling the same genes in the same way. This fact makes these adhesins a perfect candidate for identifying the processes through which *CgPDR1* hyperactivity mediates enhanced virulence.

## IV. AIM AND IMPORTANCE OF THIS STUDY

GOF mutations on *CgPDRI* not only lead to resistance to azole antifungals, but they also result in higher virulence and fitness of the affected strains (13). Results of a previous study suggest that one possible mechanism causing the higher virulence in *CgPDRI* GOF mutant strains is mediated by increased adherence to epithelial cell layers, thereby favoring epithelial colonization of the host by *C. glabrata* (15). This hypothesis is further supported by microarray data showing that adhesins, a group of cell wall proteins responsible for adherence, are consistently regulated by different GOF mutations in *CgPDRI*(24). The aim of this study is to analyze the implication of a number of known adhesins, with a focus on Epa1, Epa7, Epa12 and Pwp4, in the increased adherence of several *CgPDRI* GOF mutant strains, by means of quantitative PCR gene expression analysis. The results of this study will allow a better understanding of the mechanisms of *CgPDRI*-mediated differences in the interaction of *C. glabrata* with host cells and adherence processes of *C. glabrata* in general, possibly identifying targets for future antibiotic therapy.

## V. MATERIALS AND METHODS

### V.1. Choice of the tested adhesins

The choice of the tested adhesins has been taken by including a range of different criteria. At first the range of known adhesins has been narrowed down by including microarray data of a previous experiment of our lab, revealing the genes that are differentially regulated in *PDR1* GOF mutants (24). This selection of genes has been further narrowed down by studying the available information on the different adhesins in the available literature (17, 22, 23), leading to the inclusion of *EPA1*, the most studied adhesin to date, and an additional *EPA* adhesin, *EPA7*. *EPA6*, another well-studied *EPA*-family member has not been included because of problems with the primer design due to the high sequence homology between *EPA6* and *EPA7*. *EPA12* has been included because it showed a consistent, strong regulation in all of the studied GOF mutant strains. *PWP4* has been included to compare the expression of the *EPA* adhesins, which are located subtelomerically, to an adhesin that belongs to a different family and is not located in a subtelomeric region.

### V.2. Oligonucleotide design

A primer pair and a Taqman probe, which hybridizes to the region between the primer-specific sequences, were designed for the target genes with Geneious R7 (version 7.1.05) software (Biomatters Ltd), taking the CBS138 strain as the reference genome. *RDN5.8* was used as the reference gene with previously published primers and probe (25). All primers and probes were synthesized by Eurogentec (Table 2).

**TABLE 2, Primers and fluorescent probes used in RT PCR**

Gene	Primer / Probe	Sequence (5' – 3') and 5'/3' modifications <sup>a</sup>
<i>RDN5.8</i>	RDN5.8F	CTT-GGT-TCT-CGC-ATC-GAT-GA
	RDN5.8-R	GGC-GCA-ATG-TGC-GTT-CA
	RDN5.8-Pr	<i>FAM</i> -ACG-CAG-CGA-AAT-GCG-ATA-CGT-AAT-GTG- <i>TAMRA</i>
<i>EPA1</i>	EPA1a 95	ACC-GCA-AGA-AAA-TCC-TCC-TCC
	EPA1b 95	TGG-TGC-TGA-TGA-TAT-TGA-TTT-GTT-G
	EPA1pr 95	<i>FAM</i> -TGG-CCT-CCA-TTC-ATA-CCC-CAC-TTC-CA- <i>TAMRA</i>

<b>EPA7</b>	EPA7a 96	TGA-TTT-ACG-GAA-GAA-TGG-TTC-G
	EPA7b 96	TTA-CCG-GTA-ACA-CCA-TCA-ACT
	EPA7pr 96	<i>FAM-TGG-GAT-CTA-AAT-ATG-CGG-CAT-CCC-AAC-A-TAMRA</i>
<b>EPA12</b>	EPA12a 96	AAG-GGT-TTG-TCA-ATG-GAA-CTG
	EPA12b 96	CAC-CCT-TGG-AAA-ATT-CGG-ATC
	EPA12pr 96	<i>FAM-TCG-GAA-GAA-AGG-TTC-TCA-CCC-ATG-CT-TAMRA</i>
<b>PWP4</b>	PWP4a 96	GAG-TAG-ATC-TAG-AAC-TGC-GGG
	PWP4b 96	AGT-GAT-CAA-CTG-GGA-ACT-ACC
	PWP4pr 96	<i>FAM-ACC-CAG-CCC-TGC-AGT-GAG-TAC-TCT-TAMRA</i>

<sup>a</sup> Abbreviations: FAM, 6-carboxyfluorescein; TAMRA, 6-carboxy-N,N,N',N'-tetramethylrhodamine;

## V.1. Strains used

The *C. glabrata* strains used in this study are listed in Table 3. All strains are stored in 20% glycerol stocks at -80°C.

**TABLE 3, *C. glabrata* strains used in this study**

Strain	Background Strain	Genotype	Reference
<b>DSY562</b>	Azole-susceptible clinical strain isolated from oropharynx		(9)
<b>CBS138</b>	Clinical strain isolated from feces		CBS
<b>BG2</b>	Clinical strain isolated from vagina		(23)
<b>SFY93</b>	DSY562	<i>pdr1Δ::FRT</i>	(13)
<b>SFY114</b>	DSY562	<i>pdr1Δ::PDR1-SAT1</i>	(13)
<b>SFY115</b>	DSY562	<i>pdr1Δ::PDR1<sup>L280F</sup>-SAT1</i>	(13)
<b>SFY101</b>	DSY562	<i>pdr1Δ::PDR1<sup>R376W</sup>-SAT1</i>	(13)
<b>SFY103</b>	DSY562	<i>pdr1Δ::PDR1<sup>D1082G</sup>-SAT1</i>	(13)
<b>SFY105</b>	DSY562	<i>pdr1Δ::PDR1<sup>T588A</sup>-SAT1</i>	(13)
<b>SFY109</b>	DSY562	<i>pdr1Δ::PDR1<sup>E1083Q</sup>-SAT1</i>	(13)
<b>SFY111</b>	DSY562	<i>pdr1Δ::PDR1<sup>Y584C</sup>-SAT1</i>	(13)
<b>SFY116</b>	DSY562	<i>pdr1Δ::PDR1<sup>P822L</sup>-SAT1</i>	(13)
<b>VSY184</b>	CBS138	<i>pdr1Δ::FRT</i>	*
<b>VSY215</b>	CBS138	<i>pdr1Δ::PDR1-SAT1</i>	*
<b>VSY216</b>	CBS138	<i>pdr1Δ::PDR1<sup>L280F</sup>-SAT1</i>	*
<b>VSY250</b>	CBS138	<i>pdr1Δ::PDR1<sup>R376W</sup>-SAT1</i>	*



<b>VS251</b>	CBS138	<i>pdr1Δ::PDR1<sup>T588A</sup>-SAT1</i>	*
<b>VS249</b>	CBS138	<i>pdr1Δ::PDR1<sup>E1083Q</sup>-SAT1</i>	*
<b>VS248</b>	CBS138	<i>pdr1Δ::PDR1<sup>P822L</sup>-SAT1</i>	*
<b>VS143</b>	BG2	<i>pdr1Δ::FRT</i>	*
<b>VS144</b>	BG2	<i>pdr1Δ::PDR1-SAT1</i>	*
<b>VS145</b>	BG2	<i>pdr1Δ::PDR1<sup>L280F</sup>-SAT1</i>	*
<b>VS146</b>	BG2	<i>pdr1Δ::PDR1<sup>R376W</sup>-SAT1</i>	*
<b>VS147</b>	BG2	<i>pdr1Δ::PDR1<sup>T588A</sup>-SAT1</i>	*
<b>VS211</b>	DSY738	<i>pdr1Δ::PDR1-SAT1</i>	*
<b>VS212</b>	DSY738	<i>pdr1Δ::PDR1<sup>L280F</sup>-SAT1</i>	*
<b>VS213</b>	DSY2235	<i>pdr1Δ::PDR1-SAT1</i>	*
<b>VS214</b>	DSY2235	<i>pdr1Δ::PDR1<sup>L280F</sup>-SAT1</i>	*

\* Unpublished (Vale-Silva *et. al*)

### V.3. RNA extraction and cDNA synthesis

Overnight cultures were prepared from the desired *C. glabrata* strains in 3 mL liquid YEPD Medium. Sample preparation was performed from either two or three biological replicates. The overnight cultures were diluted in 5 mL of fresh liquid YEPD medium and grown at 30°C until mid-logarithmic phase. Subsequently, the culture tubes were centrifuged and the pellet was re-suspended in 300 µL RNA buffer (0.1 M Tris HCl pH 7.5, 0.1 M LiCl, 10 mM EDTA, 0.5% SDS) and transferred to screw top tubes. A volume of acid-washed 0.5 mm glass-beads equivalent to 200 µL and 300 µL 25:24:1 phenol chloroform isoamyl alcohol were added and the cells lysed using the MP-FastPrep (M/S 0.5, time 15 sec.). Then, tubes were centrifuged 1 min. at 14000 rpm. Following the centrifugation, 250 µL of the supernatants were transferred to clean Eppendorf tubes and mixed with 250µL 25:24:1 phenol chloroform isoamyl alcohol. After another centrifugation of 1 min. at 14000 rpm, supernatants were transferred to clean Eppendorf tubes and precipitated with 600µL chilled 100% ethanol. Tubes were vortexed for 10 s, placed on dry ice for 10 min. and then centrifuged for 2 min. at 4°C. After pouring off the supernatant, pellets were washed with ice-cold 70% ethanol and air-dried. Finally, pellets were re-suspended in H<sub>2</sub>O and RNA concentrations were determined by spectrophotometry (NanoDrop 2000, Thermo Scientific).

To remove contaminating DNA, 10 µg of each RNA preparation was treated with DNase using a DNA-free™ DNA removal kit (Ambion). For the Reverse Transcription reactions, Transcriptor High Fidelity cDNA Synthesis Kit (Roche) has been used on 1 µg of DNA-free RNA of each sample and the manufacturer's instructions were followed. RNA samples were stored at -80°C and cDNA samples were stored at -20°C.

#### V.4. Quantitative real-time PCR (qRT-PCR)

Quantitative expression of the *CgEPA1*, *CgEPA7*, *CgEPA12*, and *CgPWP4* genes was performed with the StepOne Real-time PCR System (Life Technologies). RT-PCR was carried out in a 10-µl volume containing the following reagents: 5 µl of iTaq Supermix with Rox (BioRad), each primer pair and the Taqman probe at a final concentration of 200 nM for the primers and 100 nM for the probes and 1% of the total cDNA sample produced as described above. Each reaction was run in triplicate and data was analyzed with the StepOne™ software. For relative quantification of the target genes, each set of primer pairs and the Taqman probe were used in combination with the primers and probe specific for the *RDN5.8* gene in separate reactions.

For data analysis and fold change calculations, the *Comparative CT Method* was used. For each experiment a standard curve for the reference gene and the studied gene were included and the amplification efficiency was determined for all genes. The CT values of the target genes were normalized to the endogenous reference (*RDN5.8*). Fold changes were obtained from the mean normalized expression of the samples relative to the mean normalized expression of a selected wild type (WT) control (Equation 1). A twofold increase in the level of expression of each gene was arbitrarily considered as significant.

$$\frac{X_{Test}}{X_{Control}} = 2^{\Delta\Delta C_T} = 2^{(C_{T,X}-C_{T,R})_{Control} - (C_{T,X}-C_{T,R})_{Test}}$$

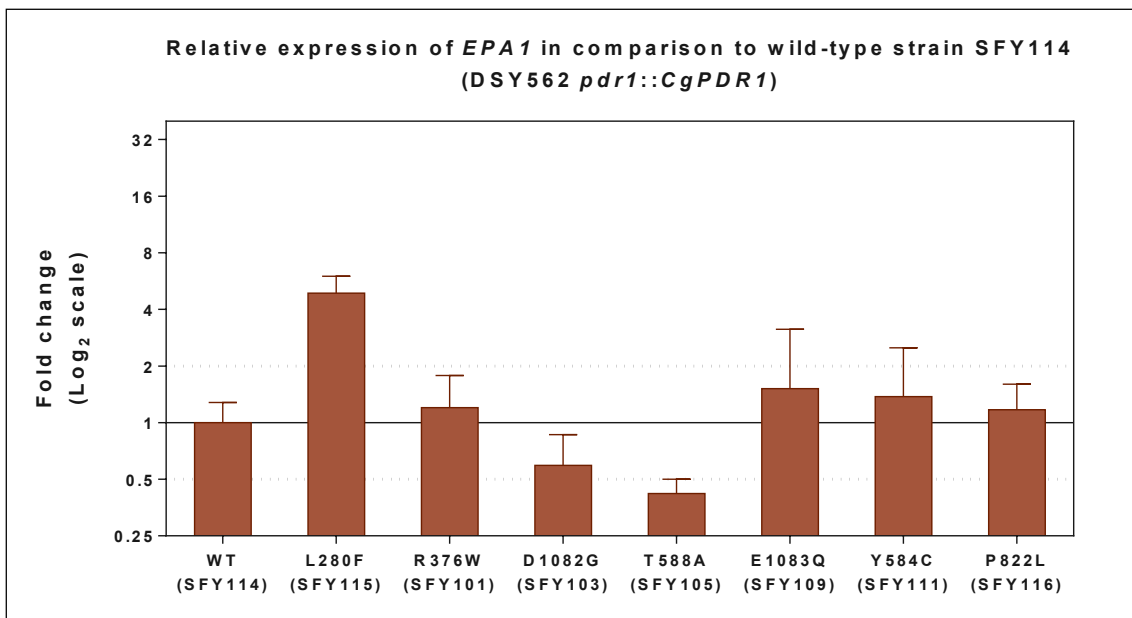
##### Equation 1

**Comparative CT Method (ΔΔCT method).** CT,X is the threshold cycle of the gene of interest and CT,R is the threshold cycle of the endogenous reference gene (*RDN5.8*). Test refers to the test cDNA sample and Control refers to the control cDNA sample.

## VI. RESULTS

### VI.1. Expression of adhesins in DSY562 background

In these experiments the expression of *EPA1*, *EPA7*, *EPA12* and *PWP4* in different GOF mutant strains in the DSY562 strain background, associated with enhanced virulence and drug resistance, has been compared to a DSY562 WT strain. The aim of this experiment was to study the influence of the different GOF mutations on the expression of the above-mentioned adhesins.



**Figure 7**

**Relative expression of *EPA1* in different *CgPDR1* GOF mutant strains compared to the wild-type strain SFY114.**

The expression of *EPA1* (Fig. 7) shows a heterogeneous pattern among the different GOF mutant strains ranging from a two-fold down regulation in the SFY105 strain (T588A mutation) to a four-fold up regulation in the SFY115 (L280F mutation) strain. The latter strain is the only one showing an up regulation of *EPA1* expression.

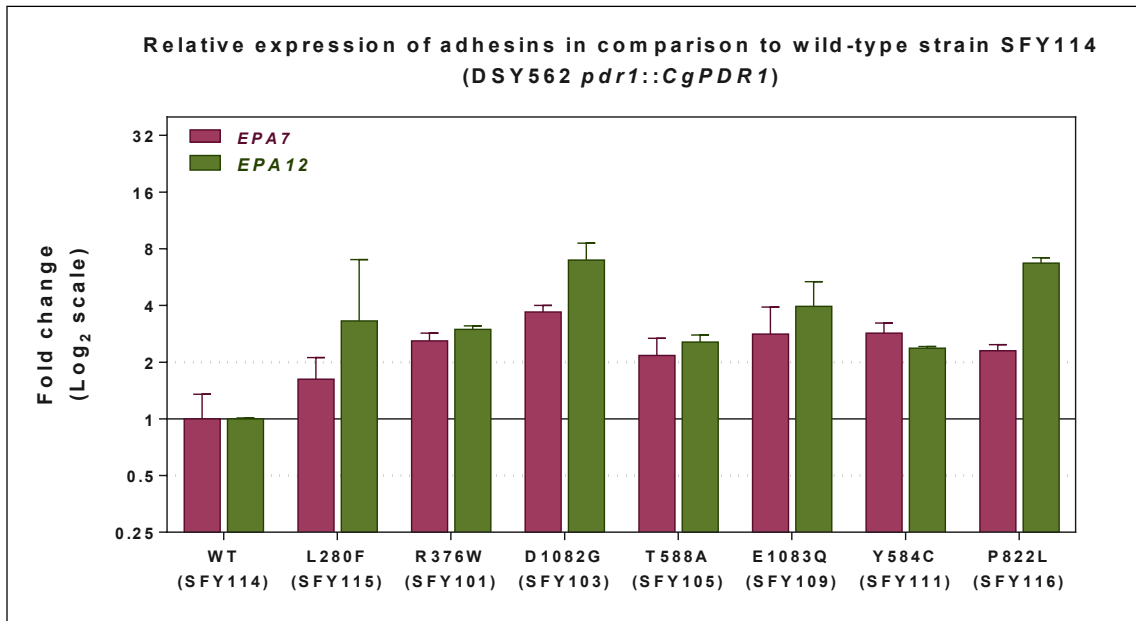


Figure 8

Relative expression of *EPA7* and *EPA12* in different *CgPDR1* GOF mutant strains compared to the wild-type strain SFY114.

*EPA7* and *EPA12* (Fig. 8) seem to have an increased expression in the different GOF mutant strains, showing a 2 to 8 fold upregulation compared to the WT strain.

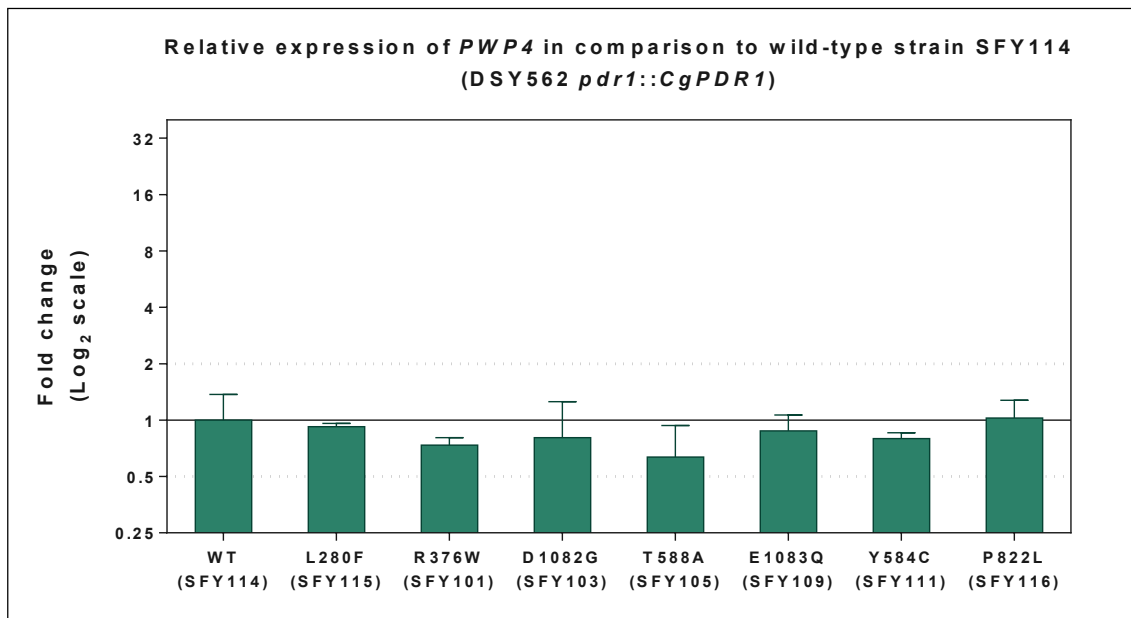


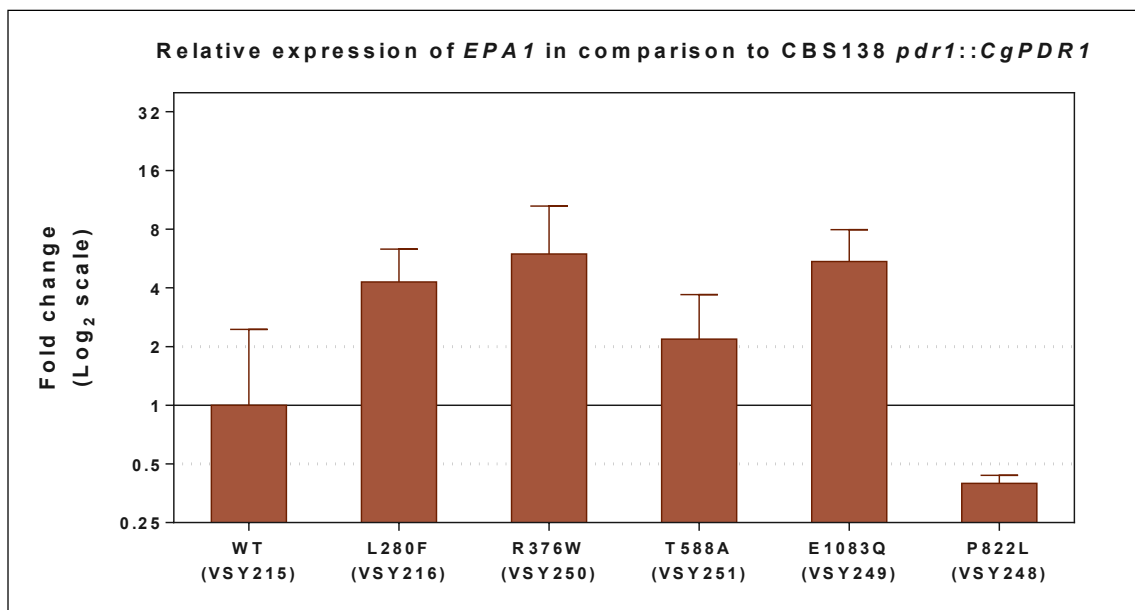
Figure 9

Relative expression of *PWP4* in different *CgPDR1* GOF mutant strains compared to the wild-type strain SFY114.

The expression of *PWP4* does not seem to be affected by the different *CgPDR1* GOF mutations and lacks a significant difference in the level of expression in comparison to the WT strain (Fig. 9).

## VI.2. Expression of adhesins in CBS138 background

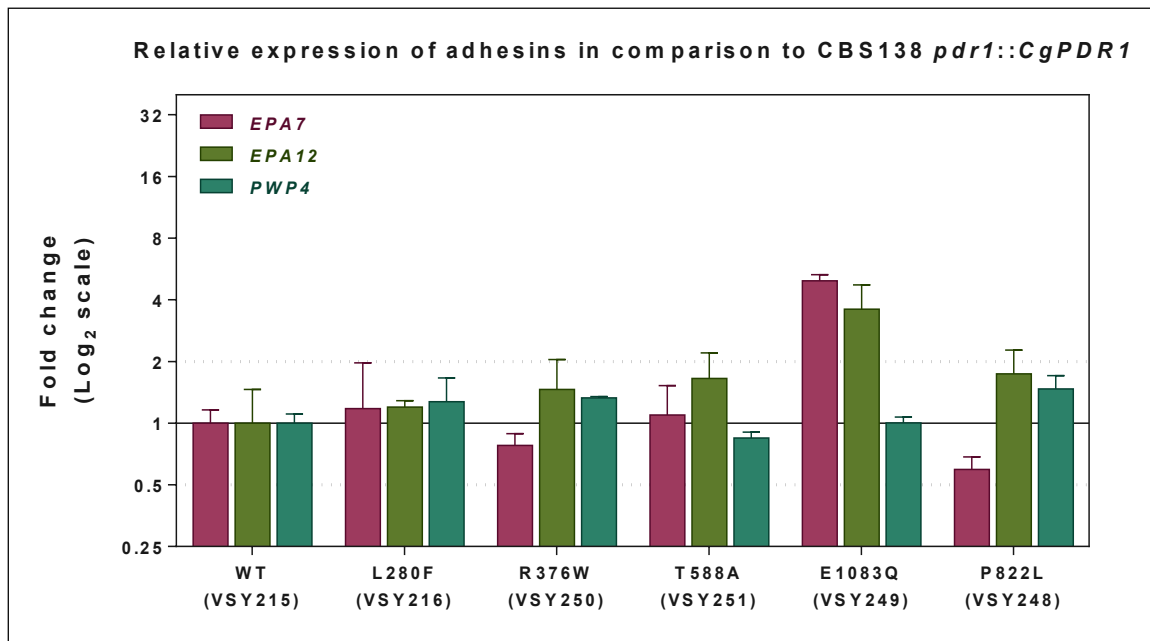
In this experiment the expression of *EPA1*, *EPA7*, *EPA12* and *PWP4* was analyzed in strains carrying *CgPDR1* alleles with the same GOF mutations as in the previous experiment but this time in the CBS138 strain background. The goal was to study the influence of the strain background on the expression levels of adhesins. A WT strain and a GOF mutant strain (L280F) on the DSY562 background have been included for comparison (data not shown).



**Figure 10**

Relative expression of *EPA1* in different *CgPDR1* GOF mutant strains compared to the wild-type strain VSY215.

*EPA1* shows at least a two-fold overexpression in all of the tested strains except for the P822L GOF mutant strain, in which *EPA1* seems to be down regulated in comparison to the WT strain (Fig. 10).



**Figure 11**

Relative expression of *EPA7*, *EPA12* and *PWP4* in different *CgPDR1* GOF mutant strains compared to the wild-type strain VSY115.

The expression levels of *EPA7*, *EPA12* and *PWP4* in the different GOF mutant strains do not show a significant change in comparison to the wild-type expression of these adhesins. The only GOF mutant strain showing an overexpression is E1083Q, presenting an overexpression of *EPA7* and *EPA12*. The *PWP4* expression, again, stays unchanged in comparison to the wild type expression level (Fig. 11).

### VI.3. Expression of adhesins in BG2 and DSY2235 backgrounds

The goal of this experiment was to study the expression of *EPA1* and *EPA7* in the available mutant strains in BG2 and DSY2235 backgrounds with an aim of analyzing the influence of the strain background on the expression levels of adhesins. Again, WT and GOF mutant strain L280F on the DSY562 background have been included for comparison (data not shown).

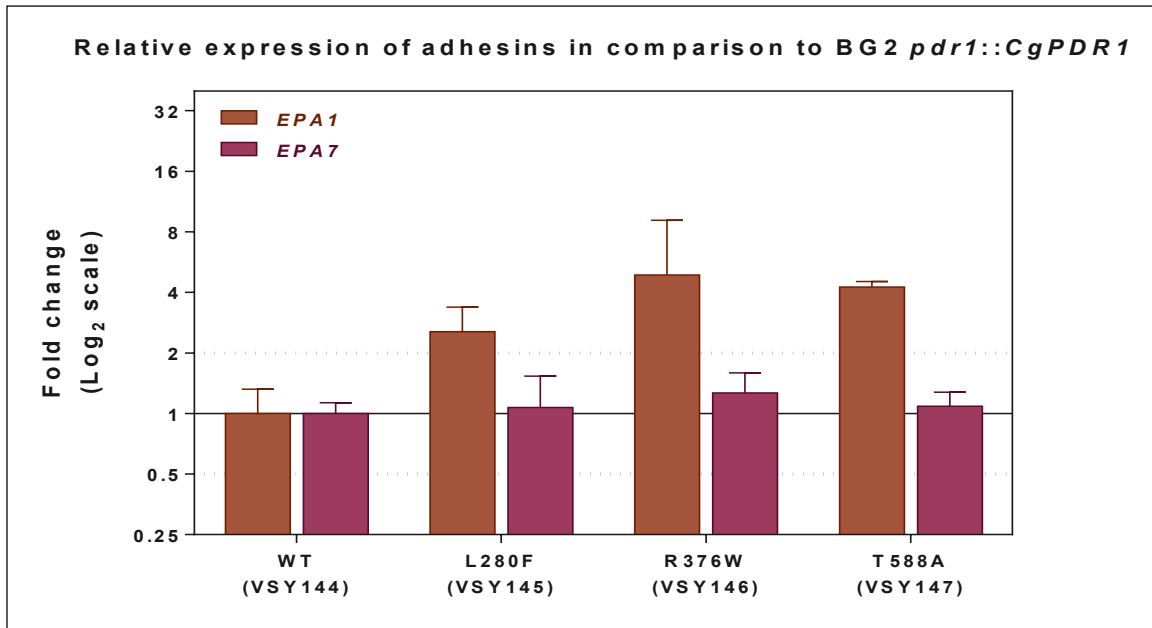


Figure 12

Relative expression of *EPA1* and *EPA7* in different GOF mutant strains in a BG2 background relative to the BG2 wild type strain VSY144.

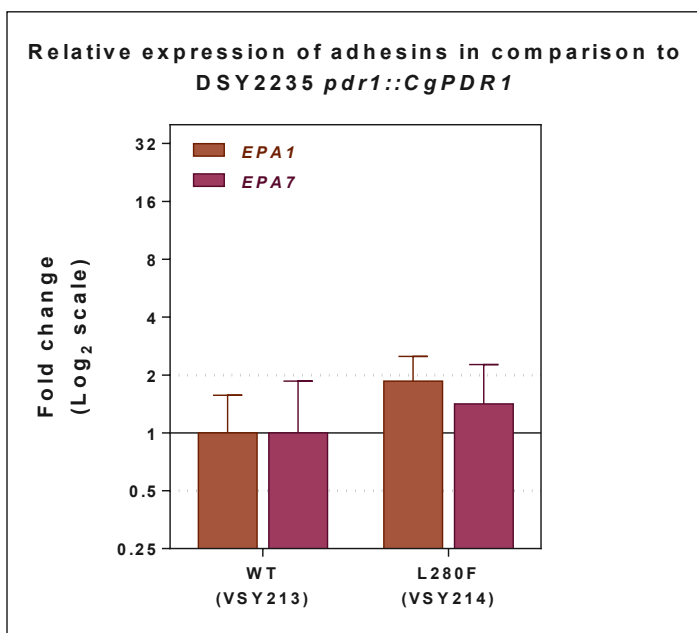


Figure 13

Relative expression of *EPA1* and *EPA7* in the L280F mutant strain in DSY2235 background compared to the DSY2235 WT strain VSY213.

*EPA1* appears to be overexpressed above the two fold threshold in all three of the studied GOF mutant strains (L280F, R376W and T588A) in the BG2 background, while the expression of *EPA7* does not seem to be affected by either of the analyzed GOF mutant strains in the BG2 background (Fig. 12). Neither *EPA1* nor *EPA7* seem to be significantly overexpressed in the L280F GOF mutant in the DSY2235 background, although *EPA1*'s relative expression is very close to the two-fold threshold (Fig. 13).

#### VI.4. Comparison of adhesin-expression in DSY562, CBS138 and BG2 backgrounds

Since we have observed substantial differences in adhesin expression between the different strain backgrounds in the previous expression experiments, we decided to compare the expression of *EPA1* in the different strain backgrounds expressing the wild type alleles of *CgPDR1* (originally from DSY562) in a single experiment.

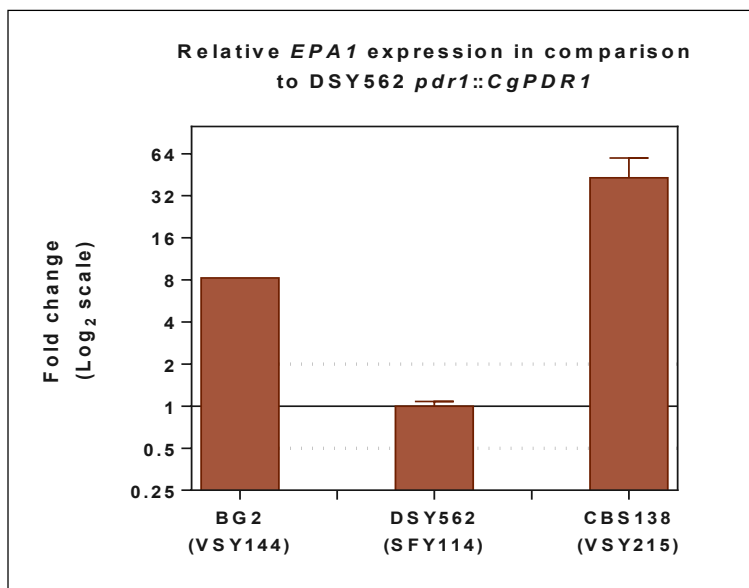


Figure 14

*EPA1* expression in BG2, DSY562 and CBS138 wild type strains relative to the DSY562 wild type strain SFY114.

Expression of *EPA1* is highest in the CBS138 strain background, lowest in the DSY562 background, and in between the two levels for the BG2 background. The difference is remarkable with over a 40-fold difference (Fig. 14) between the expression levels of *EPA1* in the DSY562 and CBS138 wild type.



## VII. DISCUSSION, CONCLUSION AND OUTLOOK

### VII.1. Regulation of adhesin expression by *CgPDR1* hyperactivity

The tested isogenic strain collections with different *CgPDR1* GOF mutations all presented an overexpression of at least one of the analyzed adhesins. This highly suggests that *CgPDR1* hyperactivity has an important influence on the expression of adhesins in *C. glabrata*. Additionally, the results vary between different *C. glabrata* strains, revealing an important influence of the genetic background.

In the DSY562 isogenic strain collection *EPA1* is only overexpressed in the SFY115 strain (L280F mutation) out of all the tested strains. *EPA7* and *EPA12*, on the other hand, are overexpressed in all the studied strains. In the CBS138 isogenic strain collection *EPA1* is the only overexpressed adhesin, while *EPA7* and *EPA12* do not seem to be affected by the *CgPDR1* GOF mutations in this strain background. The same picture presents itself for the isogenic strain collection in the BG2 strain background.

*PWP4*, in contrast to the other tested adhesins, is located in a non-subtelomeric location. Interestingly this fact correlates with the finding that *CgPDR1* GOF mutations and strain backgrounds have absolutely no influence on the expression of *PWP4*. The expression levels of *PWP4* through all the performed experiments have never exceeded the two-fold threshold level set as a limit for up or down regulation. This finding supports the idea that *CgPDR1* mediated hyper-adherence involves subtelomeric regulation mechanisms.

The differential regulation of adhesins between the tested strain backgrounds and the different GOF mutations, combined with the fact that the tested non-subtelomeric adhesin (*PWP4*) shows no regulation through *CgPDR1*, strongly points toward a complex indirect regulation of the adhesin expression through *CgPDR1*. This is further supported by the fact that subtelomeric regions are subjected to a complex regulation with a multitude of involved enzymes and regulation factors (16). However, direct regulation of at least some of the tested adhesins cannot be excluded at this point and additional work is required to confirm this theory.

## VII.2. Correlation between adherence to epithelial cells and adhesin expression

In experiments conducted simultaneously to my work at our lab, the adherence of different *CgPDR1* GOF mutation strains to mammalian epithelial cells was measured. Concerning the DSY562 strain background, the SFY115 strain (L280F mutation) showed an approximately 30% increase in adhesion to epithelial cells compared to the WT, while other GOF alleles did not lead to increased adhesion (Vale-Silva *et al.*, unpublished data). This shows a perfect correlation with the results of *EPAI* expression. *EPAI* is overexpressed in the strain bearing the L280F mutation only, while other *CgPDR1* mutations do not seem to affect it. In light of these data, *EPAI* was knocked out in strain SFY115 (L280F mutation in the DSY562 background) and this eliminated the differences in adhesion (Vale-Silva *et al.*, unpublished data). These observations strongly suggest that *EPAI* is solely responsible for the additional adhesion to epithelial cells mediated by the L280F GOF mutation on *CgPDR1* in the DSY562 background. On the other hand, *EPAI* does not seem to be implicated in the baseline adherence of these strains under the tested conditions. Other strains with different GOF mutations of the same isogenic strain collection (DSY562 background) lack significant differences in adherence to CHO-Lec2 cells and, as expected, did not show any regulation of the *EPAI* expression. These two findings combined identify *EPAI* as a possible key player of *CgPDR1* mediated adherence in at least the DSY562 background.

In the other tested strains and backgrounds (BG2 and CBS138) the situation seems to be more complex and the correlation between adherence to epithelial cells and *EPAI* expression is only partially given. Another observation made from the adherence experiments is that the different strains and backgrounds tested showed a high difference in absolute adhesion to epithelial cells ranging from 20-30% (BG2); ~50% (DSY562); ~70% (CBS138) and do not correlate entirely with *EPAI* expression levels. Together these findings suggest that the increased adherence due to hyperactivity of the *CgPDR1* GOF mutations observed in strains other than SFY115 (L280F mutation in the DSY562 background) is not only mediated by *EPAI* but also by other adhesions.

In BG2 the observed adherence was the lowest out of the three tested strain backgrounds, with just above 20% absolute adherence in the VSY145 strain (L280F

GOF mutation in BG2 background). Furthermore, the impact of an *EPA1* knockout in this strain is the highest, with an adherence that almost goes back to background levels (23). The gene expression results showed an overexpression of *EPA1* with no change of the *EPA7* expression in the GOF mutant strains. These findings imply that *EPA1* plays a predominant, if not exclusive role in the regulation of adherence in the *C. glabrata* BG2 strain, further supported by previous publications (23).

In conclusion we can say, that this present work was crucial to help identify *EPA1* as a key player in the observed increased adherence mediated by *CgPDR1* GOF mutations in our studied strains.

### **VII.3. Difficulties and limitations of the current work**

The primers for the adhesin expression analysis were designed with help of the CBS138 reference genome sequence. However, the expression analysis itself was performed on different strain backgrounds, including DSY562, BG2, DSY2235 and DSY738. We therefore took the risk of having mismatches in the sequences targeted by the primer in the different strains. We tried to minimize this risk by avoiding parts of the adhesin genes that showed a high heterogeneity between the two available genome sequences of the BG2 and CBS138 strains. Nevertheless the result of full genome sequencing of the DSY562 strain, obtained after the start of the experiments, showed several mismatches of the *EPA7* gene sequence targeted by our primers (Table 3) between the CBS138 and the newly obtained DSY562 sequence. Therefore, it cannot be excluded that the amplification efficacy of *EPA7* was suboptimal for the expression analysis in strain backgrounds other than CBS138. This is further supported by the low amplification efficacy calculated for the *EPA7* RT-qPCR expression analysis in DSY562 background strains. We did not encounter the same problem for the expression analysis of *EPA1*, *EPA12* and *PWP4*, where the targeted sequences were identical between the CBS138 and the DSY562 genome and amplification efficacy was within normal limits.

A weakness of the present work lies in the fact that *C. glabrata* was grown under laboratory conditions before RNA extraction, which only partially represents the physiological environment in which this pathogen is encountered. It is well known that environmental factors such as pH and growth phase of the yeast have a substantial

influence on the expression of adhesins in *C. glabrata* (19, 22). It would therefore be of high significance to study the impact of human epithelial cells co-cultured with the *C. glabrata* yeast on the expression of different adhesins. A murine model of infection would allow an even closer approximation of the infection mechanisms and adhesion patterns encountered in patients infected by *C. glabrata*.

#### **VII.4. Perspectives and outlook**

To obtain a clearer and more conclusive understanding of the regulation of adhesins through *CgPDR1*, it would be necessary to study the expression of further adhesins in different locations of the genome. This would additionally allow for a deeper understanding of the implication of subtelomeric mechanisms in the regulation of adherence mediated by *CgPDR1*. Likewise it would be of great help for the understanding of *CgPDR1* hyperactivity-mediated adherence to analyze additional strains with *CgPDR1* GOF mutations for their adherence properties to epithelial cells.

In order to better understand the mechanisms of how *CgPDR1* regulates the expression of adhesins it would be of high interest to investigate, as a first step, the interaction of Pdr1 with different promoter regions of *EPAI*. This would shed light on the direct interaction between Pdr1 and *EPAI*. As a next step Chromatin Immunoprecipitation (ChIP) could help to gain further insight in the pathways in which CgPdr1 interacts with its targets and eventually regulates the expression of adhesins.

In order to further study the contribution of different adhesins in the enhanced virulence mediated by *CgPDR1* hyperactivity, one could knock out different adhesins in a strain where enhanced virulence has been observed. A comparison of the virulence of this new strain with its parent strain would give us an idea of the contribution of adhesins in the gained virulence through *CgPDR1* hyperactivity.

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