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CLINICAL THERAPEUTICS

Clonal Spread of *Candida glabrata* Bloodstream Isolates and Fluconazole Resistance Affected by Prolonged Exposure: a 12-Year Single-Center Study in Belgium

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ABSTRACT *Candida glabrata* is a major cause of candidemia in immunocompromised patients and is characterized by a high-level of fluconazole resistance. In the present study, the acquisition of antifungal resistance and potential clonal spread of *C. glabrata* were explored at a single center over a 12-year period by analyzing 187 independent clinical *C. glabrata* bloodstream isolates. One strain was found to be micafungin resistant due to a mutation in the *FKS2* gene. Fluconazole resistance remained stable throughout the period and was observed in 20 (10.7%) of the isolates. An analysis of the antifungal consumption data revealed that recent prior exposure to fluconazole increased the risk to be infected by a resistant strain. In particular, the duration of the treatment was significantly longer for patients infected by a resistant isolate, while the total and mean daily doses received did not impact the acquisition of resistance in *C. glabrata*. No link between genotype and resistance was found. However, multilocus variable-number tandem-repeat analyses indicated a potential intrahospital spread of some isolates between patients. These isolates shared the same genetic profiles, and infected patients were hospitalized in the same unit during an overlapping period. Finally, quantitative real-time PCR analyses showed that, unlike that for other ABC efflux pumps, the expression of CgCDR1 was significantly greater in resistant strains, suggesting that it would be more involved in fluconazole (FLC) resistance. Our study provides additional evidence that the proper administration of fluconazole is required to limit resistance and that strict hand hygiene is necessary to avoid the possible spreading of *C. glabrata* isolates between patients.

KEYWORDS *Candida glabrata*, antifungal resistance, gene expression, genotypic identification

Candida bloodstream infection (BSI) caused by *Candida* spp. (i.e., candidemia) is the most frequent invasive fungal infection, at least in the Western world. While *Candida albicans* remains the most common cause of candidemia in the United States and Europe, *Candida glabrata* has emerged as a major pathogen during the last decades following higher azole consumption (1–5). This species is indeed less susceptible to azole drugs, while resistance to echinocandins is also increasingly reported for *C. glabrata*, narrowing the range of available agents for treatment (6–8).

Although various mechanisms involved in azole resistance in *C. glabrata* have been identified, it results mostly from the overexpression of efflux pumps that enhance the transport of azoles out of the fungal cell. These efflux pumps are notably encoded by

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TABLE 1 Antifungal susceptibility of 187 independent *Candida glabrata* bloodstream infection isolates from the University Hospitals Leuven, July 2004 to December 2015

Antifungal[a]	No. of isolates with MIC (μg/ml) of[b]:																	
	0.0039	0.0078	0.0156	0.0313	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512
AMB		2	8	14	32	38	54	38	1									
FLC									1	1	41	76	31	17	**8**	**5**	**6**	**1**
ITC		1		9	26	45	53	30	6	2		15						
VRC				1	10	53	58	38	10	4	5	8						
AFG	1	147	19	15	5													
CAS	1			80		96	10											
MFG	2	170	14				**1**											

[a]AFG, anidulafungin; AMB, amphotericin B; CAS, caspofungin; FLC, fluconazole; ITC, itraconazole; MFG, micafungin; VRC, voriconazole.
[b]Boldface type indicates MIC levels above the EUCAST breakpoints for FLC and MFG.

C. glabrata ABC transporter genes *CgCDR1*, *CgPDH1*, and *CgSNQ2*, of which the expression is mediated by the transcription factor CgPDR1 (9–13). The target of echinocandins is the enzyme synthesizing β-1,3-glucan, an important component of the fungal cell wall. A diminished echinocandin susceptibility in *C. glabrata* is caused by amino acid changes in the hot spot regions of FKS genes (i.e., *FKS1* and *FKS2*) encoding subunits of β-1,3-glucan synthase (14).

Tracing the origin of an infection is of high value for its prevention and control. Candidemia can be acquired externally, since *Candida* spp. are ubiquitous in the environment and disturbed anatomic barriers in patients may facilitate the invasion of fungal pathogens via health care workers' hands and via catheters. For *C. glabrata*, it is generally believed that the patient's own intestinal flora is the source of infection following the translocation from a previous colonization (15, 16). However, very few data are available to support this statement. Moreover, the asexual reproduction of *C. glabrata* enables clones to expand in the hospital, making some genotypes better adapted to the environment and more predominant in the population than others (17). Moreover, Healey et al. mention that a mutator phenotype caused by a mismatch repair defect is prevalent in *C. glabrata* clinical isolates (18).

The first aim of this study was to determine antifungal susceptibility among a set of clinical *C. glabrata* BSI isolates. In combination with patient antifungal exposure, the acquisition of resistance was investigated. The phylogenetic relationships between these isolates were then analyzed to reveal the possible spreading of infectious strains. Finally, the relative importance of different molecular resistance mechanisms was also estimated.

RESULTS

The characteristics of the 187 independent *C. glabrata* BSI isolates, retrospectively obtained from patients hospitalized at a 1,900-bed tertiary care center in Belgium over a 12-year period, are listed in Table S1 in the supplemental material. We noticed a significant increase in terms of annual incidence ($P < 0.05$), ranging from 0.08 episodes per 10,000 patient days (2005) to 0.53 episodes per 10,000 patient days (2013), with a mean of 0.28 per 10,000 patient days. The median age for all patients was 65 years (range, 0 to 100 years), and isolates were equally collected between male ($n = 93$) and female ($n = 94$) patients. Most isolates were obtained from patients hospitalized in the intensive care units (ICUs) ($n = 62$, 33.2%), 33 (17.6%) came from gastroenterology units, 18 (9.6%) from the emergency department, 9 (4.8%) from the burn wound unit, 9 (4.8%) from hematology units, 9 (4.8%) from the geriatric department, 9 (4.8%) from internal medicine, 6 (3.2%) from pulmonology units, 4 (2.1%) from cardiology units, 4 (2.1%) from abdominal surgery, and 24 (12.8%) from other non-ICUs.

Antifungal susceptibility and consumption. As shown in Table S1 and summarized in Table 1, the *C. glabrata* BSI isolates exhibited different levels of susceptibility to triazoles and echinocandins. The susceptibility tests revealed resistance to fluconazole (FLC) in 20 (10.7%) of the 187 isolates, while the other 167 were FLC intermediate,

TABLE 2 *Candida glabrata* bloodstream infection isolates from the University Hospitals Leuven, July 2004 to December 2015, by patient age group and frequency of fluconazole resistance

Patient age group (yrs)	No. (%) of BSI[a] isolates	
	Total[b]	FLC resistant[c]
≤1	1 (0.5)	0 (0)
2–20	2 (1.1)	2 (100)
21–25	4 (2.1)	2 (50)
26–45	13 (7)	1 (7.7)
46–55	28 (15)	3 (10.7)
56–65	47 (25.1)	6 (12.8)
66–75	53 (28.3)	5 (9.4)
76–85	25 (13.4)	1 (4)
>85	14 (7.5)	0
All ages	187 (100)	20 (10.7)

[a]BSI, bloodstream infection.
[b]Proportion to the total number of *Candida glabrata* bloodstream infection isolates, July 2004 to December 2015.
[c]Proportion to the total number of isolates in that age group. FLC, fluconazole.

denoting strains with MIC values between 0.002 and 32 μg/ml and which are considered neither susceptible nor resistant. Details on FLC resistance as a function of patients' ages are shown in Table 2. Almost 15% of patients younger than 65 years were infected with an FLC-resistant strain, in contrast to 6.5% of patients older than 65 years ($P < 0.05$). No significant increase or decrease in the trend of FLC resistance was observed ($P > 0.05$), but almost one-third ($n = 6$) of all FLC-resistant isolates were found in 2010 (Table S1). A total of 11 FLC-resistant isolates was obtained from patients who had been hospitalized in hematology units. All FLC-resistant strains showed elevated MICs for voriconazole (VRC), i.e., above the epidemiological cutoff, and 15 FLC-resistant strains had elevated MICs for itraconazole (ITC). Amphotericin B (AMB) resistance was not observed within the study population, and none of the strains exhibited multidrug resistance (Table 1 and Table S1).

Antifungal consumption data were available for 165 patients (i.e., excluding patients hospitalized at the psychiatric sites). In this group of patients, 19 patients were infected with an FLC-resistant *C. glabrata* strain, of which 15 (79%) received FLC prior to the onset of candidemia (Table S1), 1 received VRC for 23 days, and 3 were not exposed to azoles. In comparison, only 13% (19 of 146) of patients infected by an FLC-intermediate strain were previously exposed to FLC ($P < 0.05$) (Table S1), while 4 patients received VRC for 3 to 6 days. Considering the 34 patients recently exposed to FLC, the mean total doses of FLC were 10,360 mg and 7,716 mg for patients who had candidemia due to an FLC-resistant and -intermediate strain, respectively ($P > 0.05$). The difference in mean daily doses was also not significant with 659 mg and 769 mg for patients infected with an FLC-resistant strain and -intermediate strain, respectively ($P > 0.05$). However, the mean duration of recent FLC consumption (21 days) was significantly longer for patients infected with an FLC-resistant strain than for patients with candidemia due to an FLC-intermediate strain (11 days) ($P < 0.05$). After controlling for the variables with a P value of <0.2 on univariate analysis, the duration of recent FLC consumption was identified as the only significant predictor for the isolation of an FLC-resistant strain (odds ratio, 1.126496; 95% confidence interval, 1.005109 to 1.262543; $P = 0.041$).

Eighteen patients received echinocandins recently before the onset of candidemia, but the single *C. glabrata* strain (IHEM 26116) that was resistant to micafungin (MFG), isolated in 2013, was recovered from a patient that was not previously exposed to echinocandins or to any other antifungal (Table S1). Moreover, this isolate showed an atypical growth form (relatively slow growth and small colonies).

Genotyping. The loci *Cg4*, *Cg6*, *Cg10*, *VNTR6*, and *VNTR8* appeared to be polymorphic: 11 distinct alleles were found for the *Cg4* locus, 9 for *Cg6*, 13 for *Cg10*, 9 for *VNTR6*, and 9 for *VNTR8*. A cluster analysis of all 187 *C. glabrata* BSI isolates with each allele of

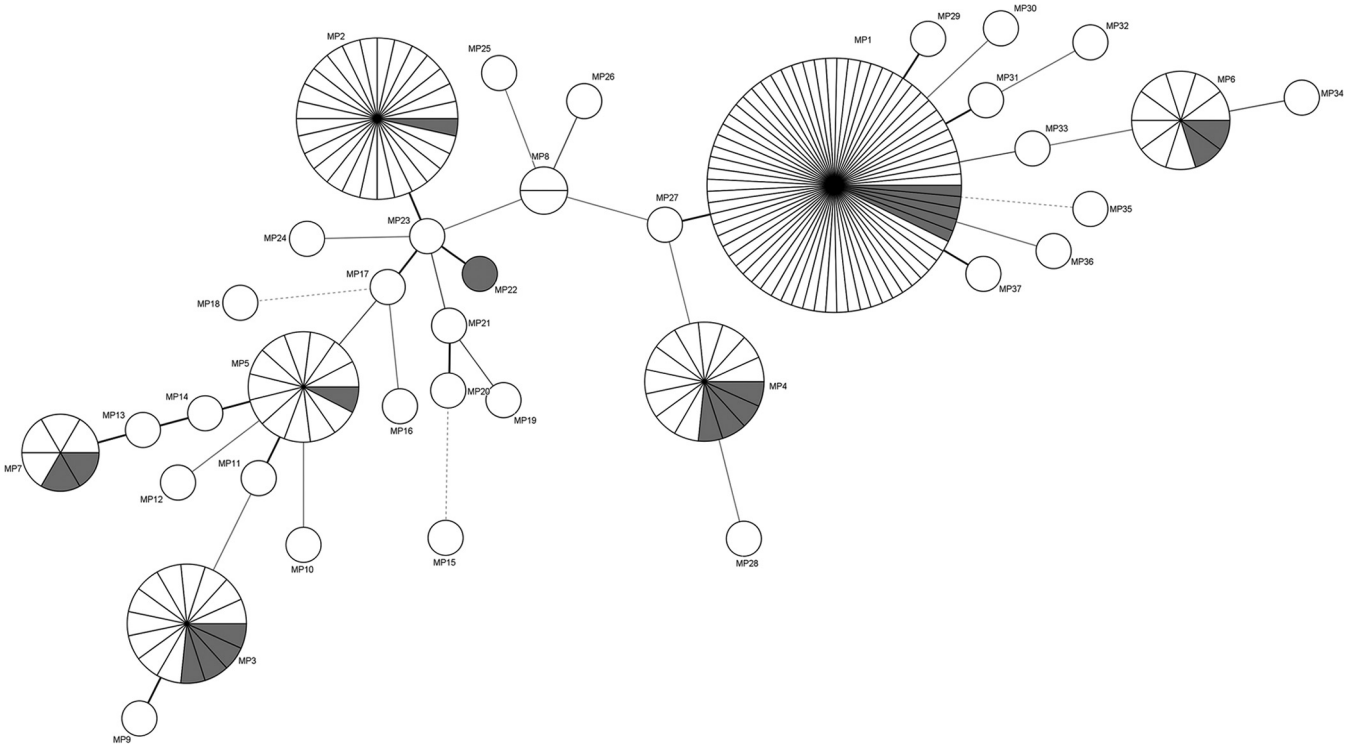

FIG 1 Minimum spanning tree. The tree displays the different genotypes (MP1 to MP37) of 187 *Candida glabrata* bloodstream infection isolates based on 5 variable-number tandem-repeat loci. The genotypes are represented by circles. The size of the circles reflects the number of isolates with that particular genotype. The length and thickness of the connecting lines between the circles show the similarity between the profiles. The fluconazole susceptibility is indicated as follows: white, intermediate; and grey, resistant.

the five microsatellite markers revealed a total of 37 different genotypes, numbered MP1 to MP37 (see Table S2). The discriminatory power was calculated between them and reached 0.82. Overall, a total of 8 distinct multilocus variable-number tandem-repeat analysis (MLVA) profiles were represented by more than one isolate. Genotype MP1 was overrepresented and occurred in 69 (36.9%) isolates. The second most frequent genotype (MP2) was distributed among 28 isolates, MP3 and MP4 occurred both in 15 isolates, followed by MP5, MP6, MP7, and MP8, shared by 13, 10, 6, and 2 isolates, respectively. The 29 remaining isolates had unique MLVA patterns (MP9 to MP37). The genetic distance between all isolates is represented by a minimum-spanning tree (MStree) (Fig. 1). By mapping the FLC susceptibility, resistant strains appeared to be scattered across the tree, indicating an absence of a genetic link between them, as confirmed by statistical analyses ($P > 0.05$). Moreover, no correlation was found between the different genotypes and one of the following characteristics: patient age, unit and year of isolation, and recent FLC consumption ($P > 0.05$).

However, 3 different clusters, each sharing the same genotype, were observed in patients with an overlapping period and hospitalization unit. In 2010, patients 66, 67, 76, 81, and 82 were admitted to the same ICU between July and September, and *C. glabrata* strains with the same MLVA profile (MP1) were found in their blood. Three of those five patients were infected with an FLC-resistant *C. glabrata* strain. During the same year, patients 73 and 80 were hospitalized in the same hematology unit during an overlapping stay and were infected with an FLC-resistant *C. glabrata* strain with genotype MP3. A similar situation occurred in 2013, when two patients (patients 136 and 146), admitted to the same ICU in September, were infected by *C. glabrata* strains with MLVA profile MP1 and similar antifungal susceptibility levels (Table S1 and S2).

FKS gene sequence analysis. The single MFG-resistant *C. glabrata* strain (IHEM 26116) was analyzed for the presence of mutations in the two hot spot regions of the

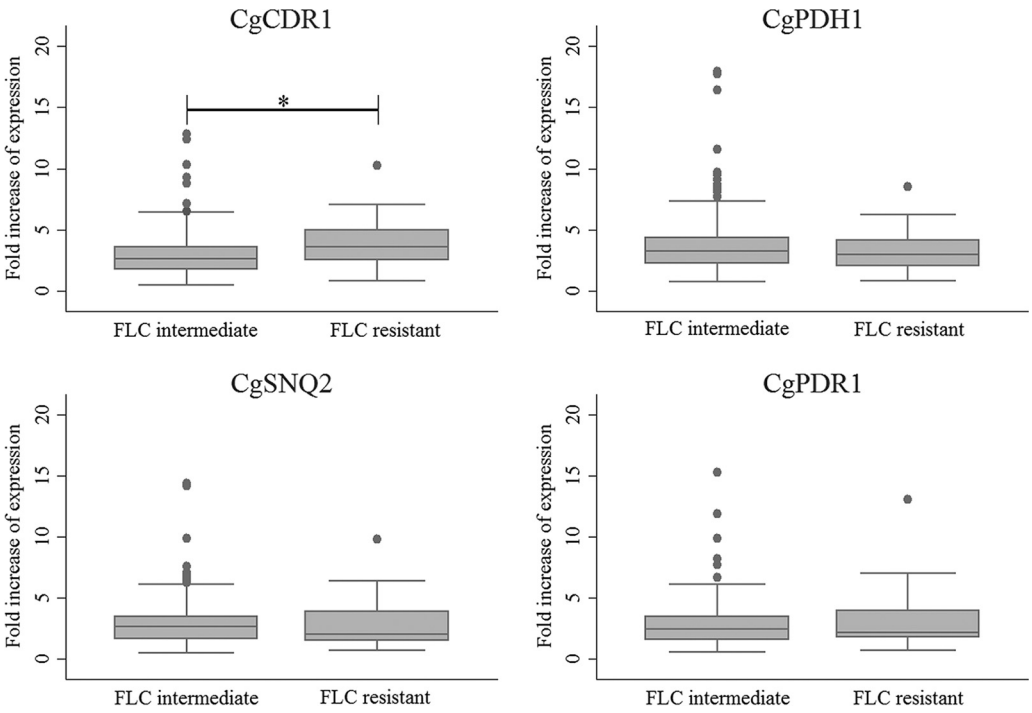

FIG 2 Gene expression. Boxplots of the fold increase in expression of *CgCDR1*, *CgPDH1*, *CgSNQ2*, and *CgPDR1* separated on the basis of fluconazole susceptibility level: fluconazole (FLC)-intermediate *Candida glabrata* bloodstream infection isolates ($n = 167$) and fluconazole-resistant isolates ($n = 20$). The analyses were performed in triplicates and results were subjected to the Mann-Whitney U test. *, $P < 0.05$.

FKS1 and *FKS2* genes. An amino acid mutation was found in the hot spot 1 region of *FKS2* and resulted in a substitution of arginine to glycine at position 665.

Gene expression analyses. Table S3 shows the results of the gene expression analyses. Eighteen of the twenty FLC-resistant isolates expressed all four efflux pump-related genes at higher levels than the FLC-intermediate control isolate IHEM 09556. The levels of expression in these isolates varied from 1.12- to 10.12-fold for *CgCDR1*, 1.24- to 8.5-fold for *CgPDH1*, 1.1- to 9.8-fold for *CgSNQ2*, and 1.2- to 13.1-fold for *CgPDR1*. Gene expression was not elevated for the 2 remaining FLC-resistant isolates, except *CgCDR1* for one of them. Considering the 167 FLC-intermediate isolates, the expression levels were as follows: 156 isolates expressed *CgCDR1* at elevated levels ranging from 1.02- to 12.79-fold, 162 raised their *CgPDH1* expression (1.03- to 17.98-fold), while *CgSNQ2* and *CgPDR1* were elevated in 158 (1.01- to 14.4-fold) and in 156 (1.01- to 15.31-fold) isolates, respectively. A simultaneous elevated expression of all four genes was observed in 149 (89.2%) of all FLC-intermediate strains. Only 2 (1.2%) FLC intermediate strains (IHEM 26411 and IHEM 26773) did not show any elevated gene expression (Table S3).

Figure 2 compares the relative expression of the four genes between the FLC-resistant and the FLC-intermediate strains. A significant difference was observed in the expression levels of *CgCDR1* between the two groups ($P < 0.05$). Moreover, *CgCDR1* expression was also significantly higher in isolates with MICs above the epidemiological cutoffs for VRC and ITC in comparison to those with lower MIC values ($P < 0.05$). In contrast, the levels of *CgPDH1*, *CgSNQ2*, and *CgPDR1* were not significantly higher for the FLC-resistant isolates than for the FLC-intermediate isolates ($P > 0.05$).

DISCUSSION

This study evaluated how *C. glabrata* candidemia is acquired and how resistance is correlated with antifungal consumption at the individual patient level by investigating the relationship within a population of *C. glabrata* BSI isolates and related patient data.

To our knowledge, this is the first time that these parameters have been analyzed in a large population (187 *C. glabrata* BSI isolates from different patients) obtained from a single hospital setting over a long-term period (12 years).

Previously, we examined the incidence of candidemia at the same center between 2004 and 2015 and reported that *C. glabrata* was responsible for almost one-fourth of infections, making it the second most common cause of candidemia, after *C. albicans* (19). Thereby, a relatively large sample size was obtained and further analyzed in this study. With the exception of one neonate patient, *C. glabrata* candidemia was absent in children less than 11 years of age, whereas half of the patients with candidemia were elderly (≥65 years). However, only a small proportion of the elderly patients was infected with an FLC-resistant strain. These results are in agreement with previous studies, confirming the high abundance but low FLC resistance of *C. glabrata* candidemia in elderly immunocompromised patients (20).

Echinocandin resistance in *C. glabrata* remains rather stable in Europe with a limited number of cases reported (21–23); this is confirmed at our center with only one case reported between 2004 and 2015. Considering FLC susceptibility, only 10% of isolates were FLC resistant, and no increase could be shown over time. This is contrary to many European and American centers where FLC resistance is increasing and can reach up to 30% (22–25). The relatively low level of FLC resistance at our center can be explained, in part, by the limited application of FLC as a prophylaxis therapy, since the latter is only provided in patients undergoing hematopoietic stem cell transplantation. Interestingly, these patients accounted for more than half of all FLC-resistant *C. glabrata* strains. FLC exposure prior to candidemia was demonstrated to select for less-susceptible species such as *C. glabrata* and to be associated with FLC resistance, especially when given at low doses (19, 26–34). Our results indicate an association between the duration of prior FLC exposure and resistance, as the mean duration of recent FLC consumption was significantly higher for patients infected with a resistant strain. This study suggests shortening, whenever possible, the duration of FLC use in order to prevent *C. glabrata* from acquiring FLC resistance.

The mutation R665G was earlier detected in a *C. glabrata* BSI isolate from a hospital in Baltimore, USA. Interestingly, this isolate was also MFG resistant but susceptible to anidulafungin (AFG) (35). Although AFG, caspofungin (CAS), and MFG belong to the same class of antifungals, it is possible that they bind to different sites on the glucan synthase complex, reflected by unique resistance mutations (14).

Multiple sources of infection have been proposed for *C. glabrata* candidemia, although the endogenous flora of the host is often considered the main source (15, 16, 36). This variability is reflected in the high number of different genotypes found in our analyses, including 29 unique profiles. However, an independent acquisition of *C. glabrata* candidemia should not be considered the only possibility. Indeed, our data confirm recent studies suggesting an epidemiological link between certain BSI isolates and indicate that infection can occasionally spread among patients. Shin et al. reported two DNA types that were shared by isolates from two patients, suggesting nosocomial transmission (37). In a Tunisian university hospital, more than one-third of *C. glabrata* invasive isolates had the same genotype, and a less common genotype was shared by 4 patients during their hospitalization in the same ICU during an overlapping time period, both indicating transmission between patients (38). Resistant *C. glabrata* strains can also be exchanged between patients as reported in other studies (8, 25). However, in our population, evidence is lacking for this type of transmission to explain the occurrence of candidemia caused by a resistant *C. glabrata* strain in the 3 patients who were not recently exposed to azoles. Disturbed anatomic barriers due to medical devices such as vascular catheters can facilitate the invasion of pathogens, and the hands of health care workers can serve as a vector of transmission. Although *C. albicans* and *Candida parapsilosis* are most frequently involved in catheter-related candidemia, these medical devices also appeared to be a possible source of infection for *C. glabrata* candidemia (39–41).

FLC resistance in *C. glabrata* is generally associated with an elevated expression of

genes encoding ABC efflux pumps (10–13, 42, 43). Previous studies investigated the importance of these molecular mechanisms but were generally limited to a set of related isolates sequentially obtained from the same patient (13, 29, 44). In contrast, we examined in a large set of independent BSI isolates whether the level of FLC susceptibility could be correlated to the expression of efflux pumps and if the latter could be associated with a specific genotype. Our results indicate the coexpression of *CgCDR1*, *CgPDH1*, and *CgSNQ2* in most of the isolates, previously attributed to their common transcription factor, CgPDR1 (11, 12, 43, 45). However, only *CgCDR1* was expressed at a significantly higher level in the FLC-resistant *C. glabrata* BSI isolates, indicating that it is the major FLC efflux pump in *C. glabrata*, consistent with previous studies (10, 41–43, 45). Although significant, the higher expression of *CgCDR1* in resistant strains was not marked and was generally lower than in previous reports (41, 42, 44), suggesting that other mechanisms are involved in FLC resistance in our set of isolates. Indeed, gain-of-function mutations in *CgPDR1* were earlier reported to mediate azole resistance in *C. glabrata* (11–13, 45), and genes other than those encoding ABC efflux pumps were shown to play a role in FLC resistance in *C. glabrata*. The latter include the *ERG11* gene, genes that belong to the major facilitator superfamily and to the aldo-keto-reductase superfamily, together with various complex interactions between different molecular mechanisms (43, 44, 46–50). Moreover, *C. glabrata* can be involved in breakthrough infections due to its capacity to exhibit inducible resistance (51). However, no significant differences in gene expression were observed in the present study between patients with a breakthrough infection and patients who did not receive azoles at the time of *C. glabrata* BSI (data not shown). Finally, no correlation was found between the level of expression and the different genotypes, suggesting that some genotypes are not prone to higher expression than others (data not shown).

Limitations of this study include the fact that it is a retrospective single-center study, although a very large sample size of 187 *C. glabrata* isolates was covered. Detailed data regarding antifungal consumption of patients during their stay at the University Hospitals Leuven between 2004 and 2015 were included, but antifungal consumption in ambulatory care was not available. Moreover, clinical information of the patients, including the type and severity of their illnesses, was not accessible. Ideally, the latter could be included in the multivariable model to know if factors other than FLC duration increase the risk of FLC-resistant *C. glabrata* candidemia. The transmission of strains suggested by MLVA could be confirmed by applying whole-genome sequencing.

In summary, our study highlights the impact of the duration of prior antifungal administration on the acquisition of FLC resistance in *C. glabrata*, emphasizing the importance of antifungal stewardship. Since isolates can be transmitted from patient to patient, the implementation of preventive strategies, such as strict hand hygiene protocols and a correct catheter care policy, is necessary to limit the potential risk of spreading. Our results favor the hypothesis that CgCDR1 is more involved than other ABC efflux pumps in FLC resistance, although it is not the only underlying molecular mechanism of FLC resistance in *C. glabrata*.

MATERIALS AND METHODS

Study population and patient data. A collection of 187 independent (i.e., originating from different patients; first isolate per episode) *C. glabrata* isolates, derived from patients suffering from candidemia at the University Hospitals Leuven over a 12-year period (July 2004 to December 2015) was obtained retrospectively. Clinical identification was confirmed by sequencing the internal transcribed spacer region. The *C. glabrata* BSI isolates were maintained at −70°C in a 10% (vol/vol) glycerol solution at the Department of Laboratory Medicine at the University Hospitals Leuven until analysis. All strains were preserved at the BCCM/IHEM culture collection (see Table S1 in the supplemental material for IHEM accession numbers). Annual and overall *C. glabrata* candidemia incidence rates were calculated on the basis of the yearly and total number of patient days, respectively (excluding patients hospitalized at psychiatric sites). The following demographic and clinical data of the patients with a *C. glabrata* candidemia were evaluated: sex, age, date at the onset of candidemia, history of hospitalization, and antifungal consumption (the latter excluding patients hospitalized at psychiatric sites).

Susceptibility testing. *In vitro* antifungal susceptibilities to the following agents were determined by applying the European Committee on Antimicrobial Susceptibility Testing (EUCAST) E.Def 7.2 guidelines: AMB, FLC, ITC, VRC, AFG, CAS, and MFG (52, 53). The concentrations of the antifungal agents ranged from

0.0078 to 16 μg/ml (AMB), 0.0313 to 1024 μg/ml (FLC), and 0.0039 to 8 μg/ml (ITC, VRC, AFG, CAS, and MFG). For FLC, *C. glabrata* BSI isolates with an MIC of >32 μg/ml were defined as resistant, while for AFG and MFG, breakpoint values were >0.06 μg/ml and >0.03 μg/ml, respectively. No breakpoint values exist for ITC, VRC, and CAS, but epidemiological cutoff values for ITC and VRC are an MIC of ≥1 μg/ml and an MIC of ≥2 μg/ml, respectively. Concerning CAS, isolates categorized as AFG susceptible can be regarded as susceptible to CAS until drug-specific breakpoints are available for CAS.

Antifungal consumption. Fluconazole and echinocandin exposure prior to the onset of candidemia was based on data extracted from the pharmacy data warehouse and considered to be any dose administered on at least 3 consecutive days within a period of 3 months before the day on which a blood sample positive for *C. glabrata* was collected. The duration of prior exposure was expressed in number of consecutive days on which any dose was given to the patient. Both the total cumulative dose (in mg) and the mean daily dose (in mg, defined as the ratio of the cumulative dose and the duration) of prior therapy were evaluated.

DNA extraction. Cultures were first freeze-dried and mechanically broken by bead-beating, followed by enzymatic digestion using proteinase K. DNA was then extracted with the Invisorb Spin Plant Minikit (Invitek) according to the manufacturer's instructions.

Genotyping. The relationship between strains was determined using MLVA. Discrimination between the isolates was obtained by using five microsatellite markers, on the basis of their high performance for studying the genetic diversity of *C. glabrata* strains, resulting in a high discriminatory power and reproducibility (17, 54–57). Each PCR was performed using 1 μl of DNA, 0.25 μM each primer, 200 μM deoxynucleoside triphosphate (dNTP) mix, 2 μl of 10× PCR buffer, 1 U of *Taq* DNA polymerase, and distilled water to a final volume of 20 μl. *Cg4*, *Cg6*, and *Cg10* were amplified by multiplex PCR using a 5′-dye-labeled primer (see Table S4). The amplification conditions were 5 min at 95°C, followed by 35 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 45 s, and a final step of 72°C for 10 min. For *VNTR6* and VNTR8, singleplex touchdown PCR was performed under the following conditions: after an initial denaturation step of 7 min at 94°C, the annealing temperature was decreased from 64 to 49°C within 15 cycles (−1°C every cycle). The cycles consisted of a 30-s denaturing step at 94°C, a 30-s annealing step (from 64°C to 49°C), and a 30-s elongation step at 72°C. After reaching a temperature of 49°C, 25 additional cycles were performed with an annealing temperature of 49°C. A dye-labeled size standard (GeneScan 400HD ROX; Applied Biosystems[ABI]) was loaded into each well along with the PCR products. The amplicon size was determined with an ABI 3130xl genetic analyzer and used to determine the number of repeats per locus. The relatedness of the strains was analyzed by an MStree analysis in Bionumerics 7.5. The discriminatory power was calculated using the Simpson index of diversity (58).

FKS sequencing. The primers used for sequencing FKS hot spot regions are listed in Table S5 (35, 59). PCR was performed with reaction mixtures containing the following reagents: 10 ng of DNA, 0.2 μM each primer, 200 μM dNTP mix, 5 μl of 10× PCR buffer, 0.5 U *Taq* DNA polymerase, and distilled water to a final volume of 50 μl (35). PCR products were purified using the Wizard PCR Preps DNA purification system kit (Promega) and sequenced with an ABI 3130xl genetic analyzer using the BigDye Terminator v3.1 sequencing kit. Nucleotide sequences were translated into amino acids with SeqMan Pro (DNAstar, Madison, WI) and aligned to detect possible mutations with BioEdit sequence alignment editor (60).

RNA extraction. Each *C. glabrata* isolate was grown on Sabouraud agar, and cells were suspended in saltwater (0.85% NaCl) to obtain a turbidity of 75%. After centrifugation, the pellets were suspended in 3 ml of liquid Sabouraud containing 1.6 μg/ml of FLC. Cultures were incubated at 37°C for 18 to 20 h, corresponding to the late-logarithmic phase as determined by growth standard curve analysis. Total RNA was extracted in triplicates using the RiboPure yeast kit (Ambion) according to the manufacturer's instructions. RNA yield and integrity were assessed by spectrophotometry and agarose gel electrophoresis. RNA samples were stored at −80°C. RNA extracts were transcribed into cDNA according to the manufacturer's instructions of the Transcriptor First Strand cDNA synthesis kit (Roche).

Quantitative real-time PCR. Quantitative real-time PCR analyses were performed to measure the expression levels of four target genes: *CgCDR1*, *CgPDH1*, *CgSNQ2*, and *CgPDR1* (see Table S6). Reactions were performed on an AriaMx real-time PCR system (Agilent Technologies) with the following reagents: 25 ng cDNA sample, 0.3 μM each primer, 12.5 μl Power SYBR green PCR master mix (ABI), and distilled water to a final volume of 25 μl. The efficiency of each PCR was determined by standard curve analysis using five 10-fold dilutions. The amplification conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, and primer annealing and elongation for 1 min at 60°C, with a melting curve analysis during the last cycle. Each experiment was carried out in triplicates, and negative controls were included in each run. The *RDN5.8* housekeeping gene was used as a reference gene to normalize the data (Table S6) (61). Comparative quantitation analyses were performed using the $2^{-\Delta\Delta CT}$ method. The fold changes were determined from the mean normalized expression of isolates relative to the mean normalized expression of a control FLC-intermediate *C. glabrata* strain (IHEM 9556) (62).

Statistical Analyses. The Mann-Kendall test was applied to investigate the trend in incidence and FLC resistance over time. A χ^2 test compared the difference between the FLC-intermediate and FLC-resistant strains concerning prior FLC exposure. Spearman's rank correlation coefficients were calculated to assess correlations between genotypes and demographic parameters and FLC susceptibility. Differences in gene expression levels between the FLC-intermediate and -resistant group and between strains with VRC and ITC MIC levels below and above cutoff values were compared with the Mann-Whitney U test.

Additional analyses were performed on patients who received FLC prior to the onset of candidemia ($n = 34$). Therefore, differences in sex, age, hospital unit, year of isolation, total dose, daily dose, and

duration of recent FLC exposure between the FLC-intermediate and -resistant groups were investigated with χ^2 tests (categorical data) and Mann-Whitney U tests (continuous data). To control for the significance of the risk factors identified in the univariate analyses, a multivariable logistic regression analysis was performed. Variables with a *P* value of <0.2 were considered for inclusion in the multivariable logistic regression analysis.

All analyses were conducted with Stata software (StataSE 14, 2013; StataCorp, College Station, TX, USA). *P* values of less than 0.05 were considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00591-18.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

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