bioRxiv preprint doi: https://doi.org/10.1101/2021.05.10.443537; this version posted May 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1

2 Reduction of susceptibility to azoles and 5-fluorocytosine and growth acceleration in

3 *Candida albicans* by glucose in urine

4

5 Running title: The effect of glucosuria on *Candida* species

6

7 Yoshiki Hiyama[a, b, c], Toyotaka Sato[b], Satoshi Takahashi[c], Soh Yamamoto[b], Noriko

8 Ogasawara[b], Naoya Masumori[a], Shin-ichi Yokota[b],

9

10 [a)] Department of Urology, Sapporo Medical University School of Medicine, Sapporo,

11 Japan

12 [b)] Department of Microbiology, Sapporo Medical University School of Medicine,

13 Sapporo, Japan

14 [c)] Department of Infection Control and Laboratory Medicine, Sapporo Medical

15 University School of Medicine, Sapporo, Japan

16

17 **Corresponding author:** Toyotaka Sato

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.10.443537; this version posted May 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 Department of Microbiology, Sapporo Medical University School of Medicine
2 S1, W17, Chuo-ku, Sapporo 060-8556, Japan
3 Telephone: +81-11-611-2111
4 E mail: sato.t@sapmed.ac.jp
5

6 **Authorship**

7 All authors meet the ICMJE authorship criteria.

8

9

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.10.443537; this version posted May 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 **ABSTRACT**

2 *Candida species* are causal pathogens for urinary tract infections,
3 vulvovaginitis, and balanitis. Diabetes mellitus is a risk factor for *Candida* infection. To
4 investigate the potential effects of glucosuria on *Candida* spp. (*C. albicans*, *C. krusei*,
5 and *C. glabrata)*, we investigated the influence of their growth and antifungal
6 susceptibilities by glucose in urine.

7 *Candida* spp. exhibited greater growth in urine with glucose (300 and 3,000
8 mg/dL) than in plain urine taken from healthy volunteers. After 24 h incubation, the
9 viable cell number was more than 10-fold higher in the urine added 3,000 mg/dL
10 glucose than in plain urine.

11 In antifungal susceptibility, more than 80% of *C. albicans* clinical isolates
12 increased minimum inhibitory concentrations of azoles (fluconazole, itraconazole,
13 voriconazole, and miconazole) and 5-fluorocytosine with the addition of glucose
14 exceeding their breakpoints. This phenomenon was not observed in clinical isolates of *C.*
15 *krusei* and *C. glabrata*. We observed the growth in the urine to which 3,000 mg/dL
16 glucose was added even in the presence of a 128-fold higher minimum inhibitory
17 concentration of fluconazole. In most of the *C. albicans* clinical isolates, the mRNA

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.10.443537; this version posted May 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 expression of the azole resistance genes *ERG11*, *CDR1*, *CDR2*, and *MDR1* increased in
2 glucose-added urine compared with plain urine.
3 In conclusion, the growth of *C. albicans* is accelerated and azoles and
4 5-fluorocytosine become ineffective as a result of a high concentration of glucose in
5 urine. These observations provide valuable information about the clinical course and
6 therapeutic effects of azoles against *C. albicans* infections in patients with diabetes
7 mellitus and hyperglucosuria.
8
9 **IMPORTANCE**
10 Diabetes mellitus is a chronic metabolic disease characterized by
11 hyperglycemia and glucosuria, with a high risk of *Candida* infection. The current study
12 demonstrated the acceleration of *Candida* growth and ineffectiveness of azoles and
13 5-fluorocytosine against *C. albicans* in urine in the presence of glucose. These
14 observations provide novel and valuable information about the clinical course and
15 antifungal treatment of *Candida* spp. in urinary tract and genital infections of diabetes
16 mellitus patients. For the treatment of urinary tract infections caused by *Candida* spp.,
17 the guidelines do not mention glucosuria. Thus, this study suggests the necessity to

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.10.443537; this version posted May 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 conduct clinical evaluations for glucosuria in patients with diabetes mellitus who have
2 urinary tract and genital infections with *Candida* spp.
3
4 **KEYWORDS**
5 *Candida* species, glucosuria, antifungal susceptibility
6
7
8
9
10
11
12
13
14
15
16
17

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.10.443537; this version posted May 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 **INTRODUCTION**

2 Diabetes mellitus is a chronic metabolic disease characterized by
3 hyperglycemia and glucosuria. Globally, an estimated 422 million people had diabetes
4 worldwide (8.5% of the adult population) in 2014 with type 2 diabetes making up about
5 90% of the cases (1). Diabetes mellitus is a condition predisposing to infections. In the
6 United States, 6 million diabetics were annually hospitalized, and 8-12% of them were
7 admitted for infection management that was responsible for over 48 billion dollars in
8 hospital charges (2).

9 Some of the cases are attributed to intrinsic infections by commensal bacteria
10 or fungi (3). Epidemiological data on the association between diabetes and infection
11 provide only limited scientific evidence that diabetes is associated with an increased risk
12 of mortality (3, 4).

13 *Candida* spp. are commensals as a part of the normal human flora, and they are
14 localized on the mucosae of the oral cavity, gastrointestinal tract and urogenital
15 apparatus, and skin. In diabetes mellitus patients, as well as susceptible hosts such as
16 elderly, hospitalized, and immunosuppressed patients, *Candida* spp. cause various
17 infections such as oral candidiasis, peritonitis, vulvovaginitis, urinary tract infections,

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.10.443537; this version posted May 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 and balanitis (5). Their colonization increases in the oral cavity and gastrointestinal tract
2 in patients with diabetes mellitus (6-8). *Candida albicans* is the most isolated fungal
3 species in urinary tract infections, and in persons with diabetes mellitus with candiduria
4 (the presence of *Candida* spp. in urine), there is a high risk of developing fungal urinary
5 tract infections (8). It is believed that candiduria is one of the causal reasons for
6 vulvovaginitis and balanitis (5). Therefore, detailed studies on candiduria and urinary
7 *Candida* infections in diabetes mellitus would be important for control and treatment.
8 However, the characteristics of *Candida* spp. in the glucosuria observed in patients with
9 diabetes mellitus have not been well elucidated.

10 In this study, we investigated the effect of glucose addition to urine on the
11 growth and susceptibility to antifungal agents of *Candida* spp. to simulate the
12 glucosuria of diabetes mellitus.

13

14

15 **RESULTS**

16 **Incidence of *Candida* spp. isolated from clinical glucosuria samples**

17 We collected 553 urine specimens (527 and 26 were from patients without and

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.10.443537; this version posted May 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 with glucosuria, respectively) from patients without duplication (Table 1). Fungi were
2 isolated from 9 (0.17%) specimens from patients without glucosuria, and all isolates
3 were *Candida* spp. *Candida* spp. were also the solo fungi isolated from 4 (15.4%)
4 specimens from patients with glucosuria. Fungi, namely *Candida* spp., were more
5 frequently isolated from urine with glucosuria than from that without glucosuria
6 (p<0.01).
7
8 **Growth of *C. albicans* in urine with various concentrations of glucose added**
9 To determine the effect of glucose in urine, the growth rate of the *C. albicans*
10 clinical isolates SMC2 and SMC40 was examined in glucose-added urine derived from
11 healthy volunteers. Addition of more than 300 mg/mL glucose to urine markedly
12 enhanced the growth (Fig. 1A and B). The viable colony count of *C. albicans* also
13 exhibited enhancement of the growth rate at a glucose concentration in urine of more
14 than 30 mg/dL (Fig. 1C). Enhancements of growth rates by the addition of glucose were
15 also observed in urine derived from other three donors (Fig. 1D).
16 We collected *Candida* spp. isolates from clinical urine specimens, and
17 investigated the fungal growth for 36 *C. albicans* (other 14 *C. albicans* isolates that had

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.10.443537; this version posted May 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 no amplification of PCR products of seven housekeeping genes for MLST analysis were
2 excluded from the evaluation.), 5 *C. krusei*, and 5 *C. glabrata* strains in urine. The 36 *C.*
3 *albicans* clinical isolates exhibited genetic heterogeneity determined by nucleotide
4 sequences obtained from MLST analysis (Fig. S1). All isolates of *Candida* spp., except
5 one strain, exhibited markedly enhanced growth with the addition of glucose at 3,000
6 mg/dL in the urine compared to the absence of glucose addition (Fig. 2). After 24 h
7 incubation, viable fungal numbers were more than 10-fold higher for *C. albicans* and *C.*
8 *glabrata*, and more than 1,000-fold higher for *C. krusei* in urine in the presence of 3,000
9 mg/dL of glucose than in plain urine (Fig. 2).
10

11 **Antifungal susceptibility in urine with various concentrations of glucose**

12 We measured minimum inhibitory concentrations (MICs) of various antifungal
13 agents in urine with various concentrations of glucose (0, 300, and 3,000 mg/dL) for 50
14 *C. albicans* clinical isolates (Fig. 3). The MICs of all azole antifungal agents examined
15 were similar in RPMI broth and urine without addition of glucose. The MICs of azoles
16 (fluconazole, itraconazole, voriconazole, and miconazole), and 5-fluorocytosine were
17 higher in urine in the presence of glucose than in plain urine (Fig. 3). The MIC levels

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.10.443537; this version posted May 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 were increased more than 128-fold for triazoles (fluconazole, itraconazole, and
2 voriconazole) and 5-fluorocytosine, and 16-fold for miconazole. The phenotypes of
3 fluconazole, itraconazole, and voriconazole were resistant. The resistance phenotypes of
4 other antifungal agents (micafungin, caspofungin, and amphotericin B) were not altered
5 in the glucose-added urine compared with RPMI broth and urine without addition of
6 glucose. These phenomena were also observed using urine from other donors (Table
7 S1).
8 The growth of *C. krusei* and *C. glabrata* in urine without addition of glucose was
9 insufficient to determine MICs of the antifungals, except for amphotericin B (Tables S2
10 and S3). In most *C. krusei* and *C. glabrata* clinical isolates, the MICs of
11 5-fluorocytosine increased, and the MICs of caspofungin decreased in urine with 3,000
12 mg/dL glucose added compared with RPMI broth. These clinical isolates did not show
13 apparent differences of the MICs of azoles, amphotericin B, and micafungin between
14 RPMI broth and urine with 3,000 mg/dL glucose (Tables S2 and S3). The MICs of
15 amphotericin B were similar or lower in urine with or without addition of glucose, than
16 with RPMI broth.
17

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.10.443537; this version posted May 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 **Time-kill assay in urine with addition of glucose**

2 We evaluated the time-dependent antifungal effect by time-kill assay in urine
3 using two *C. albicans* clinical isolates, SMC40 and SMC41 (Fig. 4). In the presence of
4 amphotericin B, which is fungicidal, at a 2-fold higher MIC, complete fungicidal
5 activity was observed within 24 h in RPMI broth, and in urine both with and without the
6 addition of glucose (Fig. 4A and C). In the presence of fluconazole, which is fungistatic,
7 at a 128-fold higher MIC, the viable cell numbers increased very slowly even after
8 2-day cultivation in RPMI broth and plain urine, and increased rapidly in urine with
9 3,000 mg/dL glucose (Fig. 4B and D). The increases in the glucose-added urine were
10 significantly higher (p<0.05) than for RPMI broth and plain urine at 12 to 48 h.
11
12 **Expression of mRNA of azole-resistant-associated genes in glucose-added urine**

13 We measured the expression of azole-resistance-associated genes (*ERG11*,
14 *CDR1*, *CDR2*, and *MDR1*) in 36 *C. albicans* clinical isolates (Fig. 5). When compared
15 with mRNA expression in the urine with and without addition of glucose, the levels
16 were increased 3.1 ± 3.7-fold for *ERG11*, 2.5 ± 1.8-fold for *CDR1*, 8.5 ± 18.6-fold for
17 *CDR2*, and 22.5 ± 34.5-fold for *MDR1* by addition of 3,000 mg/L glucose to urine.

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.10.443537; this version posted May 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1

2 **Susceptibility to antifungal agents of Mrr1-deleted *C. albicans* mutants**

3 We measured MICs of antifungal agents in the azole-susceptible *C. albicans*
4 strain SC5314 as the parent strain, and two derivatives of *MRR1*-deleted mutants
5 (SCMRR1M4A and SCMRR1M4B) (Table 2). The mutants showed constitutive
6 reduced expression of Mdr1 (9). In SC5314, the MICs of azoles and 5-fluorocytosine
7 were markedly increased in the urine by addition of glucose, whereas those of candins
8 and amphotericin B did not change. The MICs of azoles and 5-fluorocytosine against
9 the two *MRR1*-deleted mutants were also increased by the addition of 300 mg/dL
10 glucose; however, the increases MICs were smaller than those for SC5314. For instance,
11 the MICs of fluconazole against SCMRR1M4A and SCMRR1M4B were 32- and
12 64-fold lower in urine with 300 mg/dL glucose than that against SC5314. The MICs of
13 voriconazole against SCMRR1M4A and SCMRR1M4B were 2- and 8-fold lower (Table
14 2). Decreased MICs against the *MRR1*-deleted mutants compared to the parent strain
15 were also seen in urine with 3,000 mg/dL glucose.

16

17 **DISCUSSION**

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.10.443537; this version posted May 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 We observed growth acceleration of *Candida* spp. in urine induced by addition
2 of glucose in a concentration-dependent manner. This was consistent with a previous
3 study (10); however, that study was conducted by using artificial urine and only one *C.*
4 *albicans* strain. Our present study strengthened the previous observations in that: 1) the
5 fungal growth acceleration was observed in natural urine derived from healthy donors as
6 a result of adding glucose, and 2) this acceleration was observed in multiple *C. albicans*
7 clinical isolates, in addition to *C. krusei* and *C. glabrata*. We believe that our study
8 strengthens the evidence concerning the clinical risk of urinary tract infections caused
9 by *Candida* spp. under glucosuria in diabetes mellitus.

10 For the treatment of type 2 diabetes mellitus, sodium glucose transporter 2
11 (SGLT2) inhibitors such as dapagliflozin, tofogliflozin, and empagliflozin have been
12 widely used (11). SGLT2 mediates the tubular reabsorption of the majority of
13 glomerular-filtered glucose. SGLT2 inhibitors suppress glucose reabsorption via an
14 insulin-independent mechanism and thereby reduce blood glucose levels and increase
15 urinary glucose excretion (12, 13). It is known that the glucose concentration in urine of
16 patients administered SGLT2 reaches 3,000 mg/dL (14). We observed that the high
17 glucose concentration in the urine conferred markedly enhanced fungal growth of

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.10.443537; this version posted May 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 *Candida* spp. A previous study reported a tendency towards an increased prevalence of
2 *Candida* spp. in the urine of diabetic patients treated with canagliflozin (15). In addition,
3 some other studies reported increased frequencies of urinary and genital infections,
4 including vulvovaginal candidiasis and asymptomatic candiduria, during clinical trials
5 of several SGLT2 inhibitors (15-19). Therefore, these cases might be attributable to
6 fungal growth promotion in high glucosuria.

7 In the current study, we demonstrated dramatically decreased susceptibility to
8 azoles, especially triazoles, and 5-fluorocytosine of *C. albicans* in glucose-added natural
9 urine. This observation warrants great concern in the clinical setting because azoles are
10 the most commonly used agents for the treatment of urinary tract infections caused by *C.*
11 *albicans*, as well as its genital infections and vulvovaginal candidiasis. The
12 ineffectiveness of azoles was observed in urine in the presence of glucose at a
13 concentration of more than 300 mg/dL. This suggests high glucosuria in many of
14 diabetes mellitus patients because 52.5% of the patients have glucose concentrations of
15 more than 100 mg/dL in their urine regardless of SGLT2 inhibitor administration (20).
16 The azole resistance phenotype in the presence of glucose in urine was observed in
17 more than 80% of *C. albicans* clinical isolates. Therefore, high glucosuria might

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.10.443537; this version posted May 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 influence the risk of urinary and genital infections, and cause possible failure of
2 antifungal treatment of *C. albicans* cases.
3 On the other hand, *Candida* spp. other than *C. albicans*, such as *C. krusei* and
4 *C. glabrata*, also showed growth acceleration, but decreased susceptibility to antifungal
5 agents with the addition of glucose to urine was not observed. The reason for the
6 difference between *Candida* species is unknown. The biosynthesis and metabolism of
7 the fungal cell membrane ergosterol, which is the target for azoles, is likely to be
8 different among the various species. *C. albicans* can grow under 14α-sterol demethylase
9 deficiency (21). On the other hand, *C. krusei* and *C. glabrata* are intolerant to the
10 deficiency. The mechanisms of the differences, tolerance to 14α-sterol demethylase
11 deficiency and decreasing susceptibility of azoles induced by glucose, between species
12 need to be evaluated in the future.
13 To investigate the mechanism that confers the azole-resistant phenotype
14 induced by glucose, we measured the gene expression of the azole resistance genes
15 *ERG11*, *CDR1*, *CDR2*, and *MDR1*. Azoles contribute to antifungal activity by inhibiting
16 14α-sterol demethylase encoded by *ERG11*, which is involved in the biosynthesis of
17 ergosterol (22). Increased expression of Erg11 overcomes the activity of azoles and

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.10.443537; this version posted May 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 thereby increases the azole resistance level (22). Cdr1, Cdr2, and Mdr1 are efflux
2 transporters that excrete multiple compounds, including azoles. Overexpression of these
3 genes confers azole resistance (22). We found that *C. albicans* clinical isolates had
4 enhanced expression levels of *ERG11*, *CDR1*, *CDR2*, and *MDR1* in urine to which
5 glucose was added. *CDR2* and *MDR1* expression in particular increased extremely (Fig.
6 5). To confirm the contribution of Mdr1 to the azole resistance in urine, we performed
7 antifungal susceptibility tests using *MRR1*-deleted *C. albicans* mutants. Mrr1 is a
8 regulator of Mdr1, and mutations in *MRR1* result in constitutively reduced expression of
9 Mrd1 (23). The enhancement of azole resistance levels induced by glucose in urine was
10 partially inhibited in the *MRR1*-deleted mutants compared with the parent strain (Table
11 2). Thus, the azole resistance induced by glucose in urine might be partly contributed to
12 by Mrr1-dependent overexpression of the azole resistance genes.
13 In conclusion, growth acceleration in *Candida* spp. and ineffectiveness of
14 azoles and 5-fluorocytosine against *C. albicans* occur in urine in the presence of glucose
15 at high concentrations comparable to glucose concentrations in the urine of diabetes
16 mellitus patients, especially during treatment with SGLT2 inhibitors. The current results
17 provide novel and valuable information about the clinical course and antifungal

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.10.443537; this version posted May 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 treatment of *Candida* spp. in urinary tract and genital infections. For the treatment of
2 urinary tract infections caused by *Candida* spp., the guidelines do not mention
3 glucosuria (24). We thus need to conduct clinical evaluations for glucosuria in patients
4 with diabetes mellitus who have urinary tract and genital infections with *Candida* spp.
5
6
7 **Material and Methods**

8 **Collection of urine specimens and isolation of bacteria and fungi**

9 We collected the urine specimens from daily laboratory diagnostic tests in the
10 Department of Urology, Sapporo Medical University Hospital (Sapporo, Japan) in 2017.
11 This study was approved by the Sapporo Medical University Ethics Committee (No.
12 302-1031). We collected urine specimens without glucosuria and with glucosuria (with
13 a glucose concentration of more than 1,000 mg/dL) determined by using Meditape II 9U
14 (Sysmex, Tokyo, Japan). The identification of bacteria and fungi obtained by cultivation
15 tests was performed using a MALDI Biotyper (Bruker, Billerica, MA). Only the first
16 culture of one episode was investigated to avoid duplication. We calculated the
17 proportions of gram-negative and gram-positive bacteria, and fungi in samples with and

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.10.443537; this version posted May 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 without glucosuria.

2 Urine used for fungal culture experiments was obtained from healthy
3 volunteers, and filtrated with a 0.45 μm-pore membrane (TPP Filtermax, Merk KGaA,
4 Darmstadt, Germany).

5

6 **Isolation and characterization of *Candida* spp. strains**

7 We collected clinical isolates of *Candida* spp. (50 *C. albicans*, 5 *C. krusei*, and
8 5 *C. glabrata* isolates), and used them for the experiments. These isolates were isolated
9 from patients' urine at Sapporo Medical University Hospital in 2019-2020. The
10 identification of bacteria and fungi was performed using a MALDI Biotyper. Multilocus
11 sequence typing (MLST) of *C. albicans* based on seven housekeeping genes (*AAT1a*,
12 *ACC1*, *ADP1*, *MP1b*, *SYA1*, *VPS13*, and *ZWF1b*) was conducted as previously
13 described (25, 26), to confirm genetic heterogeneity of these isolates. A phylogenetic
14 tree based on the nucleotide sequence data of MLST analysis for the *C. albicans* isolates
15 was constructed based on the neighbor-joining method (27) using MEGA7 (28).

16 *C. albicans* SC5314, SCMRR1M4A and SCMRR1M4B were kindly provided
17 by Dr. Joachim Morschhäuser (9). SCMRR1M4A and SCMRR1M4B are homozygous

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.10.443537; this version posted May 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 *MRR1*Δ mutants that share decreased *MDR1* promoter activity originated from SC5314
2 (9).
3
4 **Growth curves of *C. albicans* in urine with various concentrations of glucose**
5 *C. albicans* strains, SMC2 and SMC40, were cultured on Sabouraud agar plates
6 (Nissui Pharmaceutical, Tokyo, Japan) for 24-48 h at 37°C. One colony was picked and
7 inoculated into 100 μL of urine with or without glucose in 96-well plates (VIOLAMO,
8 Osaka, Japan). The plates were cultivated at 37°C with shaking at 180 rpm. Growth
9 curves were determined by measuring values for OD_{600} at every 15 min for 25 h by
10 using an Infinite M200 PRO multimode microplate reader (Tecan, Männedorf,
11 Switzerland). Growth curves were obtained from the average of quadruple experiments.
12 To determine the viable cell number in urine culture with or without addition of
13 glucose, colony formation units (cfu) were measured. *C. albicans* at 100 cfu/mL was
14 inoculated into the urine obtained from healthy volunteers with or without glucose (30,
15 300, and 3,000 mg/dL). The urine was cultured at 37°C with shaking at 180 rpm for 1, 3,
16 6, 12, 24, 36, and 48 h. After the cultivation, series of dilutions with 0.85% NaCl were
17 spread on Sabouraud agar plates, and incubated for 24 h at 37°C, after which formed

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.10.443537; this version posted May 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 colonies were counted.

2

3 **Antifungal susceptibility**

4 MICs of antifungal agents (fluconazole, itraconazole, voriconazole, miconazole,
5 micafungin, caspofungin, amphotericin B, and 5-fluorocytosine) were determined by
6 the broth microdilution method according to Clinical and Laboratory Standards Institute
7 (CLSI) guidelines (29) using a susceptibility test kit (Eiken Chemical, Tokyo, Japan)
8 according to the manufacturer's instructions. RPMI 1640 (RPMI) broth (Eiken
9 Chemical) was used as a medium. After 24 or 48 h, the growth in each well of a 96-well
10 plate was measured by the OD_{630} value using an Infinite M200 PRO multimode
11 microplate reader. For all antifungal agents except amphotericin B, a value of less than
12 the IC_{50} [50% growth inhibition of the well of a positive control that was without any
13 agents in RPMI broth] was defined as the MIC. The breakpoints of itraconazole and
14 amphotericin B were according to EUCAST (30) because there is no definition in the
15 CLSI guidelines.

16

17 **Time-kill assay**

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.10.443537; this version posted May 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 Fluconazole and amphotericin B were purchased from FUJIFILM Wako Pure
2 Chemicals (Osaka, Japan). *C. albicans* SMC40 and SMC41 were grown overnight at
3 37°C on tryptic soy broth (TSB). Cells of each strain were added at a concentration of
4 10^5 cfu/mL to urine with or without addition of glucose and an antifungal agent. The
5 concentrations of glucose were 300 and 3000 mg/dL. The concentrations of fluconazole
6 were 16 and 64 μg/mL, which were 128-fold higher concentrations of fluconazole MICs
7 against SMC40 and SMC41, respectively. The concentration of amphotericin B was 1
8 μg/mL, which was 2-fold higher than the concentration of amphotericin B MICs against
9 both strains. The urine was incubated with shaking at 37°C. Aliquots of urine collected
10 at 1, 3, 6, 24, and 48 h were inoculated and cultured on a Sabouraud agar plate to
11 determine the viable cell numbers (cfu/mL).
12
13 **Reverse transcription-quantitative PCR (RT-qPCR)**
14 Overnight cultures of *C. albicans* clinical isolates in TSB were diluted 1:25 in
15 RPMI or urine with 3000 mg/dL glucose added, and then cultured for 3 h at 37°C. RNA
16 was isolated using Yeast Processing Reagent (Takara Bio, Shiga, Japan) and RNeasy
17 Plus mini kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions.

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.10.443537; this version posted May 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 The concentration of RNA was measured spectrophotometrically using an Infinite
2 M200 PRO. RNA (0.5 μg) was used to synthesize cDNA by utilizing ReverTra Ace
3 reverse transcription-quantitative PCR master mix with genomic DNA (gDNA) remover
4 (Toyobo, Tokyo, Japan). Expression of *ERG11*, *CDR1*, *CDR2* and *MDR1* was
5 determined as described previously (31) using KOD SYBR qPCR mix (Toyobo). The
6 PCR cycling conditions were as follows: initial activation at 95°C for 5 min, followed
7 by 40 cycles at 95°C for 10 s and 55°C for 30 s. Reactions were performed in a
8 LightCycler 480 II (Roche, Mannheim, Germany). The *ACT1* gene served as an
9 endogenous reference for normalizing expression levels. All primer sets were used as
10 previously described (31). The change in fold expression in urine with 3,000 mg/dL
11 glucose added (vs. plain urine) was calculated by the ΔΔCT method. Data are expressed
12 as the means ± standard deviations from three independent experiments.

13

14 **Statistical analysis**

15 Significant differences were determined using Fisher's exact test (Table 1), the
16 Kruskal-Wallis (Fig. 3), and Wilcoxon signed-rank test (Fig. 4). $p < 0.05$ was considered
17 significant.

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.10.443537; this version posted May 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1

2

3 **ACKNOWLEGEMENTS**

4 We thank Dr. Joachim Morschhäuser for kind provision of *MRR1*-deletd *C.*
5 *albicans* mutants. This work was supported by grants from the Japan Agency for
6 Medical Research and Development (AMED) (JP20ak0101118h0002). This work was
7 also partly supported by a grant from JSPS KAKENHI (19K18566, JP19K16648, and
8 JP20H03488). The funding sources did not play any role in the study design; in the
9 collection, analysis and interpretation of data; in the writing of the report; and in the
10 decision to submit the article for publication.

11

12

13 **Conflict of interest**

14 Satoshi Takahashi received speaker honoraria from MSD Inc., commission fee
15 from Nippon Professional Baseball Organization and Japan Professional Football
16 League, research grants from Abbott Japan Inc., Fujirebio Inc. and Roche Diagnostics
17 Inc., and scholarship contribution from Shino-Test Corporation Inc.

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.10.443537; this version posted May 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1

2

3

4

5

6 **REFERENCES**

7 1. World Health Organization. Global report on diabetes. 2016.
8 https://www.who.int/publications/i/item/9789241565257
9 2. Korbel L, Spencer JD. 2015. Diabetes mellitus and infection: an evaluation of
10 hospital utilization and management costs in the United States. J Diabetes
11 Complications 29:192-5. https://doi.org/10.1016/j.jdiacomp.2014.11.005
12 3. Peleg AY, Weerarathna T, McCarthy JS, Davis TM. 2007. Common infections in
13 diabetes: pathogenesis, management and relationship to glycaemic control. Diabetes
14 Metab Res Rev 23:3-13. https://doi.org/10.1002/dmrr.682
15 4. Bertoni AG, Saydah S, Brancati FL. 2001. Diabetes and the risk of infection-related
16 mortality in the U.S. Diabetes Care 24:1044-9.
17 https://doi.org/10.2337/diacare.24.6.1044
18 5. Rodrigues CF, Rodrigues ME, Henriques M. 2019. *Candida* sp. Infections in Patients
19 with Diabetes Mellitus. J Clin Med 8. https://doi.org/10.3390/jcm8010076
20 6. Soysa NS, Samaranayake LP, Ellepola AN. 2006. Diabetes mellitus as a contributory
21 factor in oral candidosis. Diabet Med 23:455-9.
22 https://doi.org/10.1111/j.1464-5491.2005.01701.x
23 7. Alkanani AK, Hara N, Gottlieb PA, Ir D, Robertson CE, Wagner BD, Frank DN,
24 Zipris D. 2015. Alterations in Intestinal Microbiota Correlate With Susceptibility to
25 Type 1 Diabetes. Diabetes 64:3510-20. https://doi.org/10.2337/db14-1847
26 8. Belazi M, Velegraki A, Fleva A, Gidarakou I, Papanaum L, Baka D, Daniilidou N,
27 Karamitsos D. 2005. Candidal overgrowth in diabetic patients: potential
28 predisposing factors. Mycoses 48:192-6.

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.10.443537; this version posted May 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 https://doi.org/10.1111/j.1439-0507.2005.01124.x
2 9. Morschhäuser J, Barker KS, Liu TT, Bla BWJ, Homayouni R, Rogers PD. 2007. The
3 transcription factor Mrr1p controls expression of the MDR1 efflux pump and
4 mediates multidrug resistance in *Candida albicans*. PLoS Pathog 3:e164.
5 https://doi.org/10.1371/journal.ppat.0030164
6 10. Wernli L, Bonkat G, Gasser TC, Bachmann A, Braissant O. 2013. Use of isothermal
7 microcalorimetry to quantify the influence of glucose and antifungals on the growth
8 of *Candida albicans* in urine. J Appl Microbiol 115:1186-93. http://doi:
9 10.1111/jam.12306
10 11. Tentolouris A, Vlachakis P, Tzeravini E, Eleftheriadou I, Tentolouris N. 2019. SGLT2
11 Inhibitors: A Review of Their Antidiabetic and Cardioprotective Effects. Int J
12 Environ Res Public Health 16. https://doi: 10.3390/ijerph16162965
13 12. Ghosh RK, Ghosh SM, Chawla S, Jasdanwala SA. 2012. SGLT2 inhibitors: a new
14 emerging therapeutic class in the treatment of type 2 diabetes mellitus. J Clin
15 Pharmacol 52:457-63. https://doi.org/10.1177/0091270011400604
16 13. Zhang L, Feng Y, List J, Kasichayanula S, Pfister M. 2010. Dapagliflozin treatment
17 in patients with different stages of type 2 diabetes mellitus: effects on glycaemic
18 control and body weight. Diabetes Obes Metab 12:510-6.
19 https://doi.org/10.1111/j.1463-1326.2010.01216.x
20 14. Johnsson KM, Ptaszynska A, Schmitz B, Sugg J, Parikh SJ, List JF. 2013.
21 Vulvovaginitis and balanitis in patients with diabetes treated with dapagliflozin. J
22 Diabetes Complications 27:479-84. https://doi.org/10.1016/j.jdiacomp.2013.04.012
23 15. Nicolle LE, Capuano G, Ways K, Usiskin K. 2012. Effect of canagliflozin, a sodium
24 glucose co-transporter 2 (SGLT2) inhibitor, on bacteriuria and urinary tract
25 infection in subjects with type 2 diabetes enrolled in a 12-week, phase 2 study. Curr
26 Med Res Opin 28:1167-71. https://doi.org/10.1185/03007995.2012.689956
27 16. Nyirjesy P, Zhao Y, Ways K, Usiskin K. 2012. Evaluation of vulvovaginal symptoms
28 and *Candida* colonization in women with type 2 diabetes mellitus treated with
29 canagliflozin, a sodium glucose co-transporter 2 inhibitor. Curr Med Res Opin
30 28:1173-8.
31 17. Musso G, Gambino R, Cassader M, Pagano G. 2012. A novel approach to control
32 hyperglycemia in type 2 diabetes: sodium glucose co-transport (SGLT) inhibitors:
33 systematic review and meta-analysis of randomized trials. Ann Med 44:375-93.
34 https:// doi: 10.3109/07853890.2011.560181

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.10.443537; this version posted May 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 18. Wilding JP, Woo V, Soler NG, Pahor A, Sugg J, Rohwedder K, Parikh S. 2012.
2 Long-term efficacy of dapagliflozin in patients with type 2 diabetes mellitus
3 receiving high doses of insulin: a randomized trial. Ann Intern Med 156:405-15.
4 https://doi.org/10.7326/0003-4819-156-6-201203200-00003
5 19. Gadzhanova S, Pratt N, Roughead E. 2017. Use of SGLT2 inhibitors for diabetes and
6 risk of infection: Analysis using general practice records from the NPS MedicineWise
7 MedicineInsight program. Diabetes Res Clin Pract 130:180-185.
8 https://doi.org/10.1016/j.diabres.2017.06.018
9 20. Gong S, Guo J, Han X, Li M, Zhou L, Cai X, Zhu Y, Luo Y, Zhang S, Zhou X, Ma Y, Ji
10 L. 2017. Clinical and Genetic Features of Patients With Type 2 Diabetes and Renal
11 Glycosuria. J Clin Endocrinol Metab 102:1548-1556.
12 https://doi.org/10.1210/jc.2016-2332
13 21. Shimokawa O, Nakayama H. 2000. Estimation of minimum sterol
14 14alpha-demethylation-inhibitory concentration of azoles in *Candida* yeasts using
15 acetate-mediated growth inhibition: potential utility in susceptibility testing. J Clin
16 Microbiol 38:2893-6. https://doi.org/ 10.1128/JCM.38.8.2893-2896.2000
17 22. Kontoyiannis DP, Lewis RE. 2002. Antifungal drug resistance of pathogenic fungi.
18 Lancet 359:1135-44. https://doi.org/10.1016/S0140-6736(02)08162-X
19 23. Dunkel N, Blass J, Rogers PD, Morschhäuser J. 2008. Mutations in the multi-drug
20 resistance regulator MRR1, followed by loss of heterozygosity, are the main cause of
21 MDR1 overexpression in fluconazole-resistant *Candida albicans* strains. Mol
22 Microbiol 69:827-40. https://doi.org/10.1111/j.1365-2958.2008.06309.x
23 24. Pappas PG, Kauffman CA, Andes DR, Clancy CJ, Marr KA, Ostrosky-Zeichner L,
24 Reboli AC, Schuster MG, Vazquez JA, Walsh TJ, Zaoutis TE, Sobel JD. 2016.
25 Clinical Practice Guideline for the Management of Candidiasis: 2016 Update by the
26 Infectious Diseases Society of America. Clin Infect Dis 62:e1-50.
27 https://doi.org/10.1093/cid/civ933
28 25. Bougnoux ME, Morand S, d'Enfert C. 2002. Usefulness of multilocus sequence
29 typing for characterization of clinical isolates of *Candida albicans*. J Clin Microbiol
30 40:1290-7. https://doi.org/10.1128/jcm.40.4.1290-1297.2002
31 26. Tavanti A, Gow NA, Senesi S, Maiden MC, Odds FC. 2003. Optimization and
32 validation of multilocus sequence typing for *Candida albicans*. J Clin Microbiol
33 41:3765-76. https://doi.org/10.1128/jcm.41.8.3765-3776.2003
34 27. Saitou N, Nei M. 1987. The neighbor-joining method: a new method for

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.10.443537; this version posted May 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 reconstructing phylogenetic trees. Mol Biol Evol 4:406-25. https:// doi:
2 10.1093/oxfordjournals.molbev.a040454
3 28. Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics
4 Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol 33:1870-4 https:// doi:
5 10.1093/molbev/msw054
6 29. Clinical and Laboratory Standards Institute : Reference Method for broth dilution
7 antifungal susceptibility testing of yeasts : Approved standard-third edition M27-A3.
8 CLSI, Wayne, PA, USA, 2008.
9 30. European Committee on Antimicrobial Susceptibility Testing Breakpoint tables for
10 interpretation of MICs for antifungal agents Version 10.0, valid from 2020-02-04.
11 https://eucast.org/astoffungi/clinicalbreakpointsforantifungals.
12 31. Flowers SA, Barker KS, Berkow EL, Toner G, Chadwick SG, Gygax SE,
13 Morschhäuser J, Rogers PD. 2012. Gain-of-function mutations in UPC2 are a
14 frequent cause of ERG11 upregulation in azole-resistant clinical isolates of *Candida*
15 *albicans*. Eukaryot Cell 11:1289-99. https://doi.org/10.1128/EC.00215-12
16

1 **TABLE 1. Microorganisms isolated from urine of patients positive and negative for glucosuria**

2

Glucosuria [a]	Bacteria		Fungi
	Gram-negative	Gram-positive	*Candida* spp.[b]
Negative (527)	299 (56.7%)	354 (67.2%)	9 (0.17%)
Positive (26)	17 (65.4%)	19 (73.1%)	4 (15.4%)**

3

4 a, Glucosuria was determined by using Meditape II 9U. Positive: >1,000 mg/dL of glucose.

5 b, included *C. albicans*, *C. glabrata*, *C. krusei*, and the *C. parapsilosis* group.

6 **p <0.01 vs. Negative.

7

8

TABLE 2. Minimal inhibitory concentrations of antifungal agents against *MRR1*-deleted *C. albicans* mutants

Antifungal agents	SC5314 (parent strain)			SCMRR1M4A (*MRR1*Δ::FRT/ *MRR1*Δ::FRT SC5314 clone A)			SCMRR1M4B (*MRR1*Δ::FRT/ *MRR1*Δ::FRT SC5314 clone B)		
	RPMI	Urine + 300	Urine + 3000	RPMI	Urine + 300	Urine + 3000	RPMI	Urine + 300	Urine + 3000
FLCZ	0.25	64	>64	0.25	1	>64	0.5	2	>64
ITCZ	0.06	8	>8	0.06	0.12	2	0.12	0.12	4
VRCZ	< 0.015	0.25	>8	0.03	0.03	>8	0.03	0.12	>8
MCZ	<0.03	0.5	2	0.06	0.5	0.5	0.06	0.5	0.12
5-FC	<0.12	8	2	<0.12	8	8	<0.12	8	2
MCFG	<0.015	<0.015	<0.015	<0.015	<0.015	<0.015	<0.015	<0.015	<0.015
CPFG	0.25	0.12	0.25	0.25	0.25	0.25	0.25	0.25	0.25
AMPH-B	0.5	0.25	0.25	0.5	0.25	0.25	0.5	0.25	0.5

Values are MICs (mg/L). FLCZ, fluconazole; ITCZ, itraconazole; VRCZ, voriconazole; MCZ, miconazole; 5-FC, 5-fluorocytosine; MCFG, micafungin; CPFG, caspofungin; AMPH-B, amphotericin B.

Urine + 300, urine added 300mg/dL glucose; Urine + 3000, urine with 3,000mg/dL glucose added.

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.10.443537; this version posted May 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 **Figure legends**

2

3 **FIG 1. Growth rates of *C. albicans* in urine with various concentrations of glucose**
4 **added**
5 (A) Growth curves of *C. albicans* SMC2 in urine with glucose added were determined
6 by measuring the values for the optical density at 600 nm. (B) The turbidity of *C.*
7 *albicans* SMC40 culture at 48 h in urine with glucose added. (C) Growth determined
8 by viable cell numbers (cfu/mL) of *C. albicans* SMC2 in urine with glucose. (D)
9 Growth curves of *C. albicans* SMC40 culture in urine derived from three other donors,
10 adding glucose, and RPMI 1640 medium.
11

12 **FIG 2. Viable cell numbers of clinical isolates of *C. albicans* (n=36), *C. krusei* (n=5),**
13 **and *C. glabrata* (n=5) in urine with (3,000 mg/dL) or without addition of glucose**
14 **after 24 h cultivation**
15 Viable cell numbers were determined by cfu/mL.

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.10.443537; this version posted May 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 **FIG 3. Minimal inhibitory concentrations (mg/L) of antifungal agents against *C.***
2 ***albicans* clinical isolates (n=50) cultured in RPMI1640 (standard procedure),**
3 **urine, and urine added glucose at 300 and 3,000 mg/dL.**
4 **, p <0.01
5
6 **FIG 4. Time-kill assay of amphotericin B and fluconazole in *C. albicans* clinical**
7 **isolates, SMC40 (A and B) and SMC41 (C and D)**
8 SMC 40 and SMC41 were cultured in RPMI 1640, urine, and urine to which 3,000
9 mg/mL glucose was added in the presence of amphotericin B or fluconazole.
10 Concentrations of antifungals were 2-fold the MIC for amphotericin B (1 mg/L for
11 both SMC40 and SMC41), and 128-fold higher the MIC for fluconazole (16 mg/L for
12 SMC40 and 64 mg/mL for SMC41). The MICs were determined using RPMI 1640
13 medium. Viable cell numbers were determined as cfu/mL. *, p <0.05, **, p <0.01
14
15 **FIG 5. Effects of glucose on mRNA expression levels of *ERG11, CDR1, CDR2* and**
16 ***MDR1* in *C. albicans* clinical isolates (n=36) during culture in urine and urine with**
17 **3,000 mg/dL glucose added**

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.10.443537; this version posted May 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

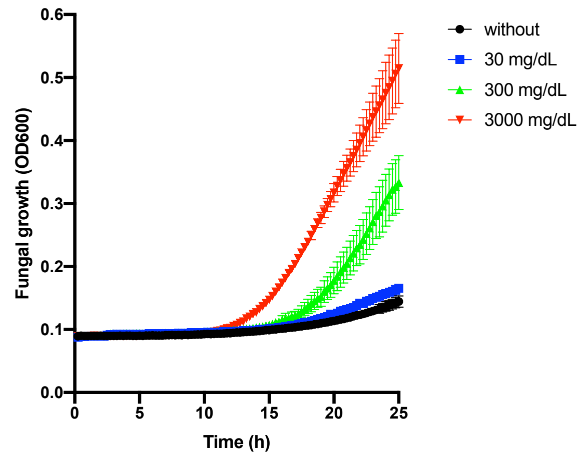
1 The expression levels were shown by relative expression level (-fold) compared with
2 the levels of culture in plain urine.
3
4
5 **Supplemental data**
6
7 **Table S1. Minimal inhibitory concentrations of antifungal agents against *C.***
8 ***albicans* SMC40 in RPMI1640 (standard procedure) and urine derived from**
9 **different donors in the presence (300 and 3,000 mg/mL) and absence of glucose**
10 **(Excel file)**
11
12 **Table S2. Minimal inhibitory concentrations of antifungal agents against *C. krusei***
13 **isolates in RPMI1640 (standard procedure) and urine in the presence (3,000**
14 **mg/mL) and absence of glucose (Excel file)**
15

bioRxiv preprint doi: https://doi.org/10.1101/2021.05.10.443537; this version posted May 11, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

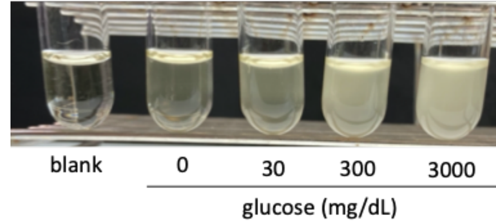
1 **Table S3. Minimal inhibitory concentrations of antifungal agents against *C.***
2 ***glabrata* isolates in RPMI1640 (standard procedure) and urine in the presence**
3 **(3,000 mg/mL) and absence of glucose (Excel file)**
4
5 **FIG S1. Phylogenetic tree based on MLST analysis in *C. albicans* clinical isolates**
6 **(n=36)**

FIG 1

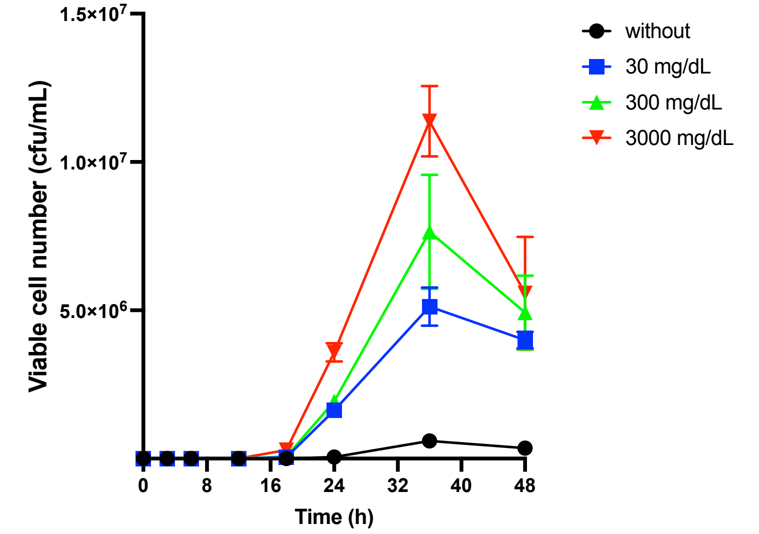
A



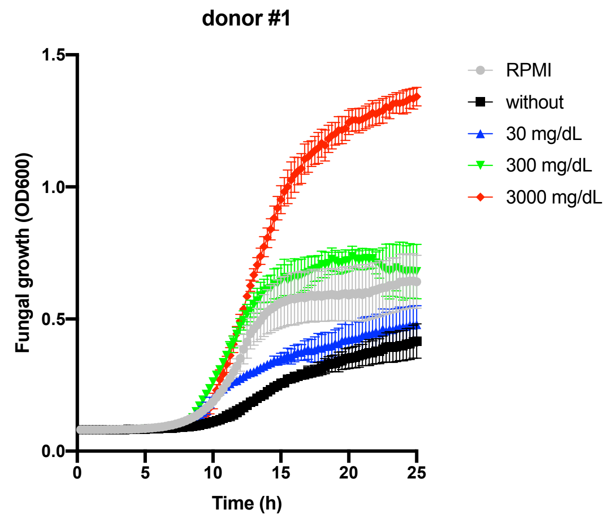
B

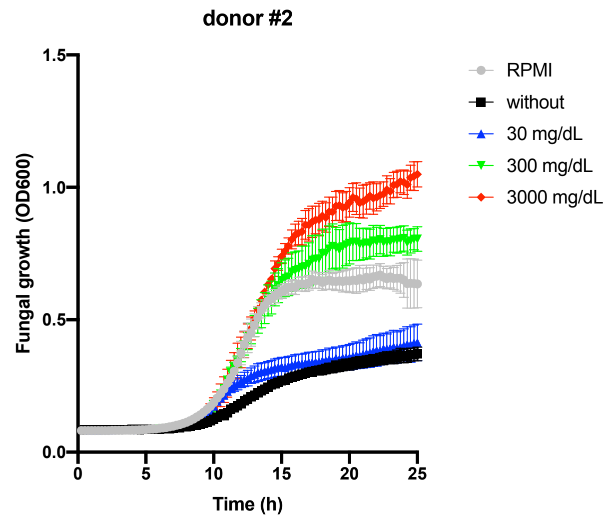


C



D





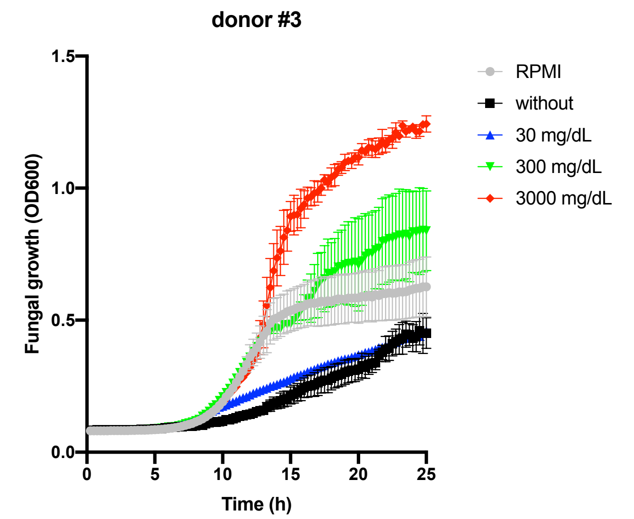


FIG 2

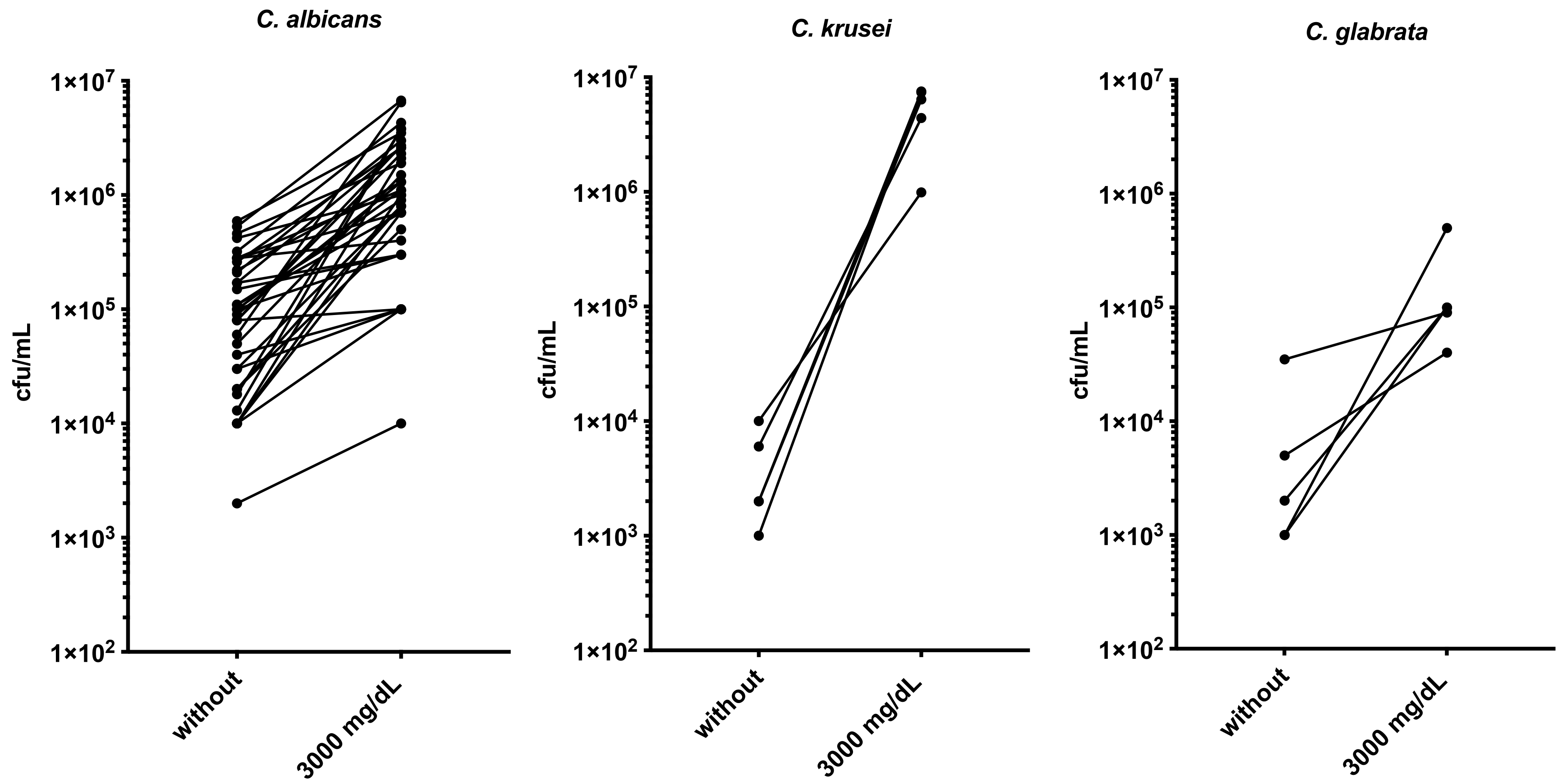


FIG 3

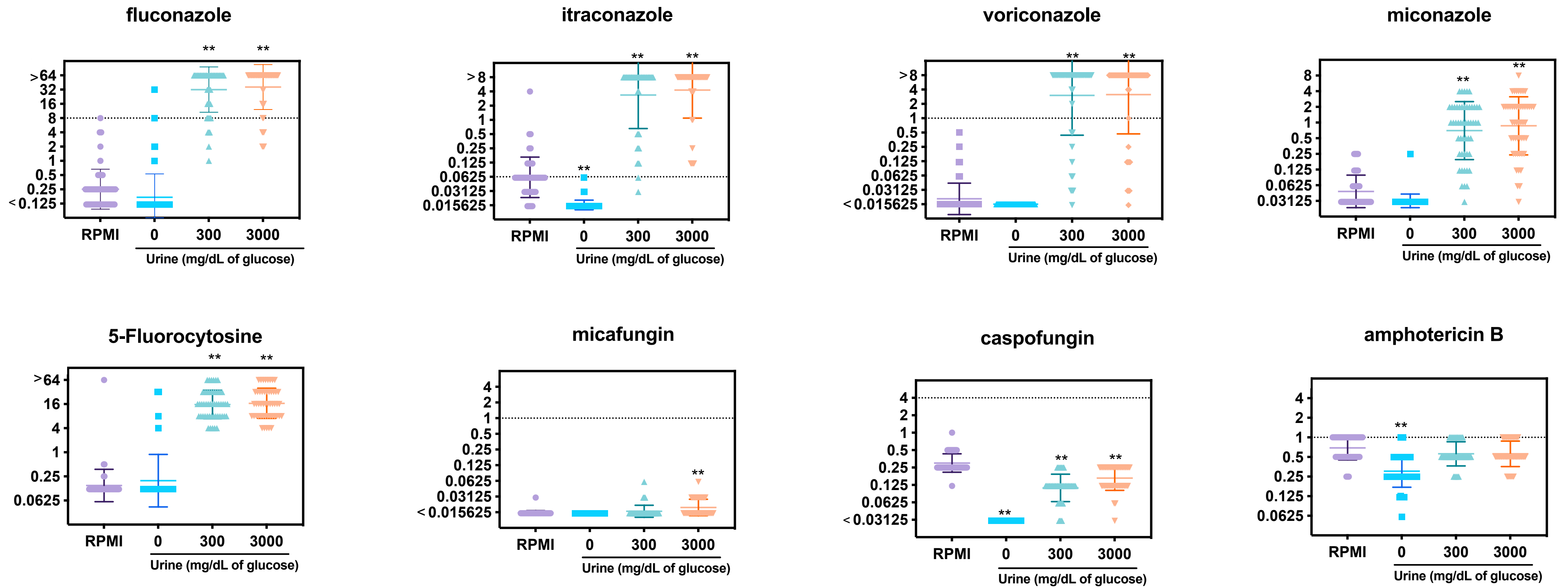


FIG 4

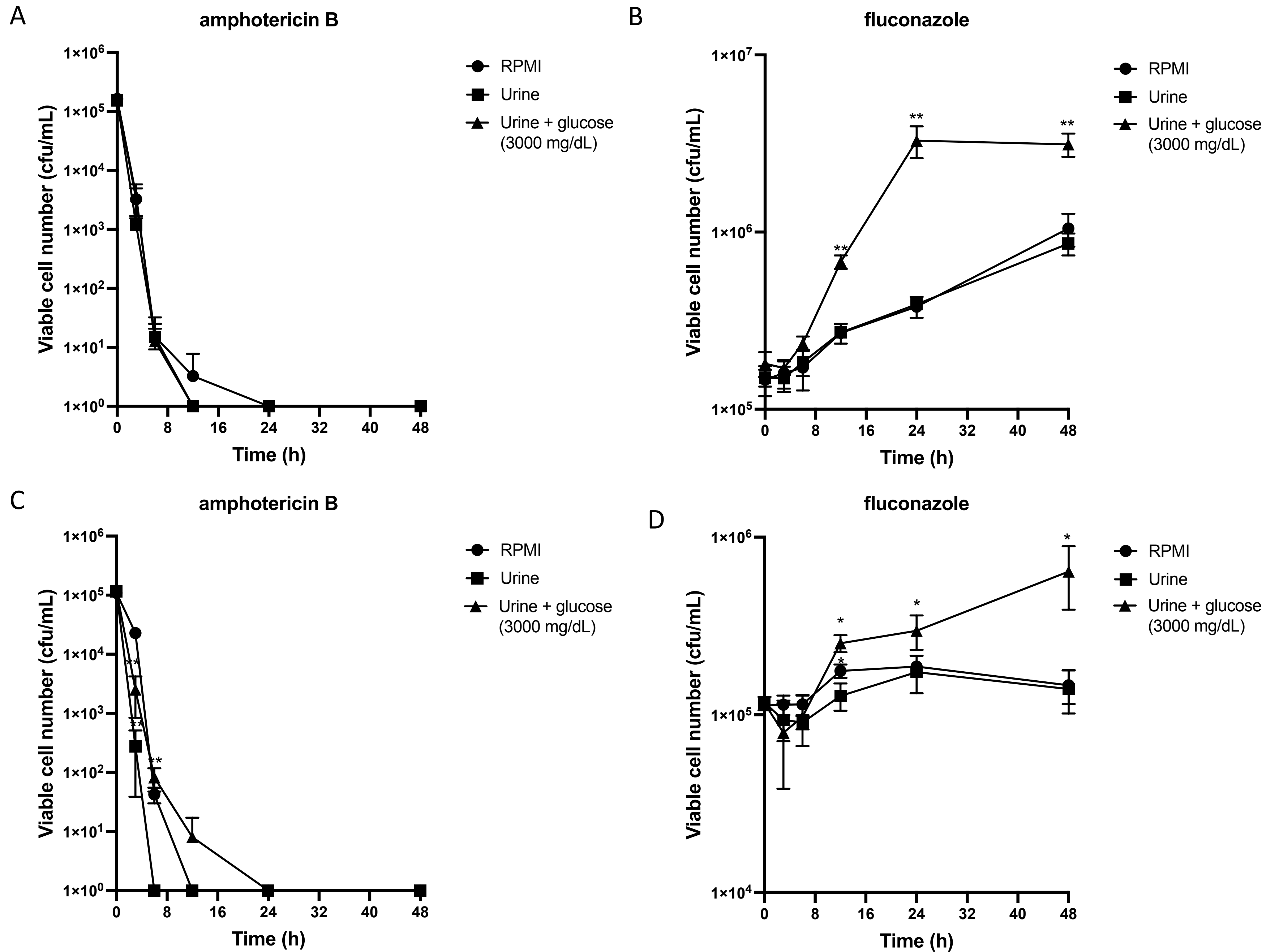


FIG 5