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Candida biofilm production is associated with higher mortality in patients with candidaemia

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Summary

Background: Candidaemia is a common life-threatening disease among hospitalised patients, but the effect of the *Candida* biofilm-forming ability on the clinical outcome remains controversial.

Objective: The aim was to determine the impact of biofilms, specifically focusing on biofilm mass and metabolic activity, on the mortality in candidaemia.

Patients/Methods: The clinical data of patients (n = 127) treated at the University of Debrecen, Clinical Centre, between January 2013 and December 2018, were investigated retrospectively. Biofilm formation was assessed using the crystal violet and XTT assays, measuring the biofilm mass and metabolic activity, respectively. Isolates were classified as low, intermediate and high biofilm producers both regarding biofilm mass and metabolic activity. The susceptibility of one-day-old biofilms to fluconazole, amphotericin B, anidulafungin, caspofungin and micafungin was evaluated and compared to planktonic susceptibility.

Results: Intermediate/high biofilm mass was associated with significantly higher mortality (61%). All *Candida tropicalis*, *Candida parapsilosis* and *Candida glabrata* isolates originating from fatal infections were intermediate/high biofilm producers, whereas this ratio was 85% for *Candida albicans*. Solid malignancy was associated with intermediate/high biofilm producers (P = .043). The mortality was significantly higher in infections caused by *Candida* strains producing biofilms with intermediate/high metabolic activity (62% vs. 33%, P = .010). The ratio of concomitant bacteraemia was higher for isolates forming biofilms with low metabolic activity (53% vs 28%, P = .015).

Conclusions: This study provides evidence that the *Candida* biofilms especially with intermediate/high metabolic activity are related to higher mortality in candidaemia.

KEYWORDS

biofilm, bloodstream infections, *Candida*, metabolic activity, mortality, susceptibility

1 | INTRODUCTION

Candidaemia is the fourth and sixth most common bloodstream infection among hospitalised patients in the United States of America and Europe, respectively, accounting for 8%-15% of all nosocomial bloodstream infections.[1-4] Candidaemia episodes are associated with unacceptably high overall mortality ranging from 38% to 75%.[5-7] A population-based surveillance from Italy revealed that biofilm production in *Candida* isolates is significantly associated with central venous or urinary catheter use and administration of total parenteral nutrition in the host patient.[8] Biofilm formation is a key virulence factor for *Candida* species; it serves as a focus in bloodstream infections, protects the fungal cells against the immune response; furthermore, the presence of biofilms is associated with reduced susceptibility to antimicrobial agents.[9] Until recently, data on the clinical impact of *Candida* biofilm on mortality were scarce and there were more contradictions than answers due to the diverse experimental settings and variable patient populations.[10-12] Several studies drew attention to the problems possibly arising from the variability of biofilm detection methods and the poorly standardised cut-off points to distinguish biofilm-producer isolates from strains with low biofilm production capability.[10-13] Generally, two simple rapid screening platforms are used to examine biofilm production, that is quantification of biomass production using crystal violet-based staining[14] and quantification of the metabolic activity of sessile cells using XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) reduction assay.[15,16] Most studies addressing the association between fungal biofilms and patient parameters characterise biofilm production using either of the two approaches; however, these two measurements are not interchangeable, that is a biofilm with high biofilm mass may show low metabolic activity or vice versa. Consequently, it is possible that some clinical parameters are differently associated with the biofilm-related variables.

Nonetheless, the association of clinical variables with biofilm mass and metabolic activity at the same time is less studied.[12] Hence, reports on the association of *Candida* biofilms with mortality are frequently contradictory[8,10-13]; several studies showed that biofilm production is linked to significantly higher mortality,[8,10] while other results do not support this observation.[11,12] In this study, we used XTT and crystal violet assays to simultaneously assess the clinical impact of biofilm development on mortality, in patients with candidaemia.

2 | MATERIALS AND METHODS

2.1 | Patients and definitions

One hundred and twenty-seven *Candida* isolates collected between January 2013 and December 2018 at 127 candidaemia episodes from the 1667-bed University Clinical Center in Debrecen, Hungary, were studied. An episode of candidaemia was defined as the isolation of *Candida* spp. from at least one blood culture in a patient. If *Candida* spp. was isolated more than once from the same patient, positive blood cultures at least 30 days apart were considered two different candidaemia episodes. Patients with multiple *Candida* spp. from the same episode were excluded. Data on patient demographics, underlying medical conditions and details of antimicrobial therapy were collected through review of the medical history. Concomitant bacteraemia was defined as isolation of potentially pathogenic bacteria from the same blood culture sample set. Patient outcomes were followed from the first positive blood culture until 30 days or death.

2.2 | Isolate identification and storage

All isolates were identified using matrix-assisted laser desorption/ionisation/time-of-flight (MALDI/TOF) analysis. Briefly, single colonies from freshly grown isolates from Sabouraud dextrose agar were spread onto ground-steel target plate in duplicates. Afterwards, cells were macerated by adding 1 μL formic acid solution. After drying, 1 μL of matrix solution (trans-Cinnamic acid dissolved in a 50:47.5:2.5 acetonitrile:water:trifluoroacetic acid) was added to each spot and then the plate was air-dried at room temperature. Mass spectra were generated with Microflex Biotyper (Microflex, Bruker Daltonics) using the manufacturer's standard settings. Mass fingerprints were acquired using FlexControl version 3.0 software (Bruker Daltonics), analysed over a mass range from 2000 to 20 000 Dalton and compared with the Bruker Daltonics database. Following the identification, isolates were stored in liquid bouillon with 15% glycerine at −20°C until further use.

2.3 | Biofilm formation

Biofilms were prepared as described previously.[15] One-day-old biofilm mass was quantified with the crystal violet assay; metabolic activity was examined with the XTT assay as previously described by O'Toole (2011)[14] and Hawser et al (1996),[16] respectively. The crystal violet and XTT measures of biofilm formation do not necessarily correlate and can be considered two independent characteristics of a biofilm.[13,17] Therefore, isolates were categorised either based on their level of biomass or based on metabolic activity distribution as low, intermediate or high biofilm producers according to a study published by Rajendran et al (2016).[10] Isolates within the first quartile (Q1) were classified as low biofilm producers, isolates with a biomass higher than the third quartile (Q3) were classified as high biofilm formers and those in between were classified as intermediate biofilm producers (Q2).[10] The principle of this classification was also followed for categorisation based on metabolic activity. For better comparability with similar studies, isolates were compared as follows: low versus intermediate/high biomass and low versus intermediate/high metabolic activity.

2.4 | Susceptibility testing of planktonic cells

Antifungal susceptibility of *Candida* isolates to fluconazole (Sigma), amphotericin B (Sigma), caspofungin, micafungin and anidulafungin (all from Molcan) was determined using the broth microdilution method in RPMI-1640 (with L-glutamine and without bicarbonate, pH 7.0 with MOPS; Sigma), using the CLSI standard M27-A3 protocol.[18] The final concentrations of the drugs were 0.03-32 mg/L, 0.016-8 mg/L and 0.008-4 mg/L for fluconazole, amphotericin B and the three tested echinocandins, respectively. All isolates were measured in triplicate, and medians were used for further analysis. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used as quality control strains in each experiment. Planktonic minimum inhibitory concentrations (MICs) were read visually following a 24-hours incubation period at 35°C. For fluconazole and the tested echinocandins, the partial inhibition criterion was used (at least 50% growth reduction as compared with the growth control), while for amphotericin B, the total inhibition criterion (100% growth reduction as compared with the growth control) was used.[18]

2.5 | Susceptibility testing of biofilms

The activity of tested antifungals against biofilms was assessed using the XTT assay.[19-21] The concentrations tested in MIC determination against biofilms were 0.06-512 mg/L, 0.015-8 mg/L and 0.008-4 mg/L for fluconazole, amphotericin B and the tested echinocandins, respectively. For *C parapsilosis*, the tested echinocandin concentrations ranged from 2 to 1024 mg/L due to the previously well-documented intrinsic FKS mutation.[22] To determine the sessile MICs, the biofilms were washed three times with 200 μL sterile physiological saline to remove unadhered cells. All wells were filled with 100 μL 0.5 g/L XTT in 1 μmol/L menadione solution. The plates were covered with aluminium foil and incubated at 35°C for 2 hours. After incubation, 80 μL of the supernatant was removed and transferred to a new sterile 96-well plate to measure the absorbance spectrophotometrically at 492 nm. MICs were defined as the lowest concentration that caused at least 50% reduction in metabolic activity of the biofilms for all antifungals. The percentile change in metabolic activity caused by drug exposure was calculated as followed: $100 \times [(A_{well} - A_{background})/(A_{drug\text{-}free\ well} - A_{background})]$, where *A* is the absorbance measured. The background was measured from the fungus-free well.[19-21] All isolates were tested in three independent experiments, and the median of the three values was used for further analysis. In each biofilm-related experiments, *C albicans* SC 5314 reference strain was used as the quality control.

2.6 | Statistical analysis

The biofilm mass and the metabolic activity of biofilms by different *Candida* species were analysed using the Kruskal-Wallis test with Dunn's post-test. The differences in MIC values against given antifungals for planktonic and sessile cells were analysed using the Wilcoxon matched-pairs tests. Fisher's exact test was used to investigate whether any of the risk factors in patients predisposed to infection with biofilm-former isolates and to evaluate whether biofilm production of the isolates had any relationship with 30-day mortality. The effect of biofilm production (logarithmised absorbance values with XTT or crystal violet) as well as the different patient characteristics and conditions on patient mortality was analysed in multivariate logistic regression models. Data were analysed using GraphPad Prism 6.05 software or in R. The results were considered significant if the *P*-value was <.05.

2.7 | Ethical approval

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. This study was approved by the institutional ethics committee (University of Debrecen, Regional and Institutional Research Ethics Committee [DE RKEB/IKEB], permission number 5190-2019).

3 | RESULTS

The majority of patients were male (59%), with a mean age of 61 years. Seventy-nine per cent of examined patients was treated in the intensive care unit. *Candida albicans* was the most prevalent species; it accounted for 51% (65/127) of examined candidaemia episodes, followed by *C parapsilosis* (23/127; 18%), *Candida tropicalis* (19/127; 15%), *C krusei* (10/127; 8%), *Candida glabrata* (4/127; 3%) and other less common species (6/127; 5%) *Candida lypolitica*, *Candida catenulata*, *Candida guilliermondii*, *Candida dubliniensis*, *Candida inconspicua* and *Candida orthopsilosis* each causing one episode. Figure 1A,B show that biofilm mass and the metabolic activity of sessile cells by different *Candida* species were heterogeneous, irrespective of the species examined. *C tropicalis* isolates had significantly higher biofilm mass compared with other *Candida* species ($P < .001$-$.05$); furthermore, their metabolic activity was significantly higher compared with *C glabrata* and *C krusei* ($P < .01$-$.05$; Figure 1A,B), but not as compared to other species.

Biofilm mass produced by various *Candida* isolates was evaluated using the crystal violet assay. Isolates were categorised as low, intermediate or high biofilm producers if their absorbance was less than Q1 ($OD_{540\ nm} = 0.01$), in between Q1 and Q3 ($OD_{540\ nm} = 0.01$-0.276) or higher than Q3 ($OD_{540\ nm} = 0.276$), respectively. Fifteen, 42 and 8 *C albicans*; 8, 7 and 8 *C parapsilosis*; 12, 7 and 0 *C tropicalis*; 4, 6 and 0 *C krusei*; and 1, 3 and 0 *C glabrata* were defined as low, intermediate and high biofilm producers, respectively. Concerning less common species, the tested *C catenulata* and *C inconspicua* isolates were low biofilm producers, while

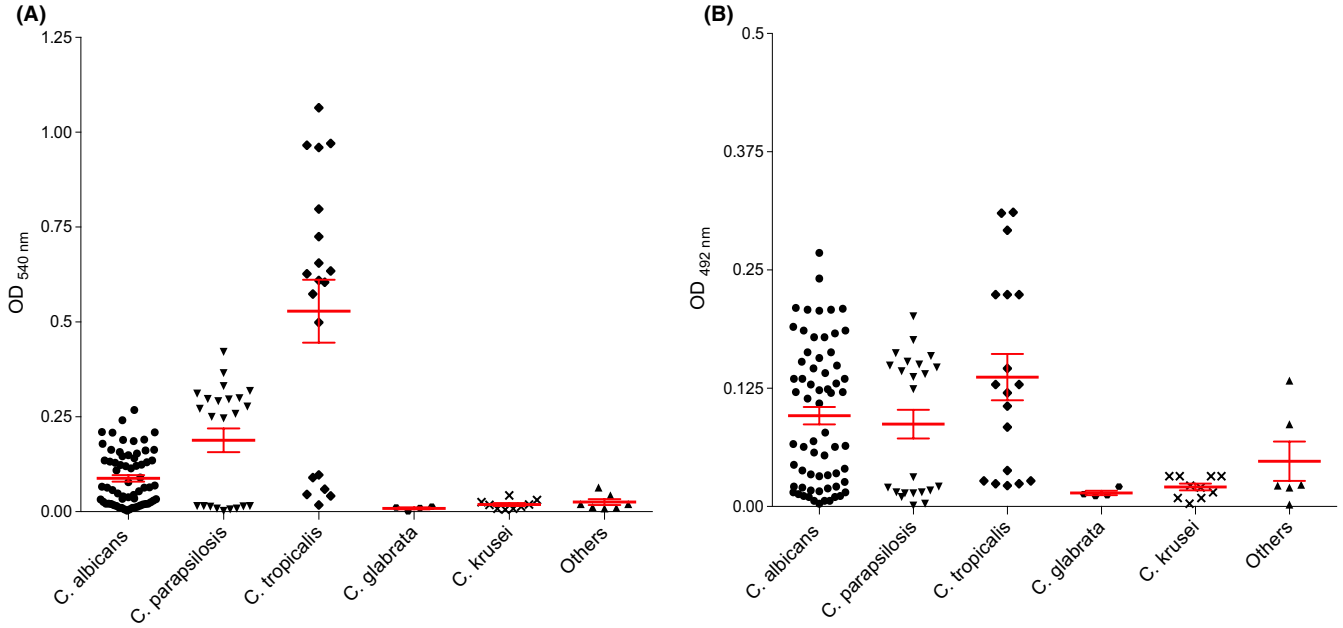

FIGURE 1 Biofilm formation of different *Candida* species by crystal violet (A) and XTT (B) assays. Biomass and metabolic activity were quantified spectrophotometrically by reading absorbance at 540 nm and 492 nm, respectively. Three replicates were used for each isolate with the mean of each represented. *C tropicalis* isolates showed significantly higher biofilm mass compared with other *Candida* species ($P < .001$-0.05) (A); furthermore, their metabolic activity was significantly higher compared with *C glabrata* and *C krusei* ($P < .01$-0.05) (B)

C lypolitica, *C guilliermondii*, *C dubliniensis* and *C orthopsilosis* isolates were intermediate biofilm producers.

Similar to the crystal violet-based investigations, we categorised the various sessile *Candida* isolates based on their metabolic activity using the XTT reduction assay. We considered the isolates with low, intermediate or high metabolic activity if their absorbance was less than Q1 ($OD_{492\ nm}$ = 0.019), Q1-Q3 ($OD_{492\ nm}$ = 0.019-0.149) or higher than Q3 (OD_{492nm} = 0.149), respectively. Fourteen, 34 and 17 *C albicans*; 8, 8 and 7 *C parapsilosis*; 0, 13 and 6 *C tropicalis*; 4, 6 and 0 *C krusei*; and 3, 1 and 0 *C glabrata* were defined with low, intermediate and high metabolic activity, respectively. The tested *C catenulata* has low metabolic activity, while *C inconspicua*, *C lypolitica*, *C guilliermondii*, *C dubliniensis* and *C orthopsilosis* isolates have intermediate metabolic activity.

The median planktonic MIC values of isolates ranged from 0.125 to >32 mg/L, 0.06 to 1 mg/L, 0.008 to 2 mg/L, 0.03 to 1 mg/L and 0.008 to 2 mg/L for fluconazole, amphotericin B, anidulafungin, caspofungin and micafungin, respectively. Based on revised CLSI breakpoints, fluconazole resistance was observed for 3 *C albicans* and all *C krusei*.[23] Furthermore, the one obtained *C inconspicua* isolate had decreased susceptibility to fluconazole (MIC = 8 mg/L). All fluconazole-resistant *C albicans* strains, 8 *C krusei* isolates and one obtained *C inconspicua* showed low biofilm mass and low metabolic activity. Concerning echinocandin resistance, 19 *C albicans*, 6 *C tropicalis*, 6 *C krusei* and 3 *C glabrata* strains showed intermediate susceptibility against caspofungin; isolates resistant to caspofungin or with decreased susceptibility to other echinocandins were not found.[24] Three out of these nineteen *C albicans* isolates showed intermediate/high biofilm mass and intermediate/high metabolic activity. This ratio was 83%, 20% and 67% for *C tropicalis*, *C krusei* and *C glabrata*, respectively.

Sessile MIC values were determined in cases of biofilms produced by tested *C albicans* and non-albicans isolates with intermediate and high metabolic activity as determined according to the above described quartile-based classification (Figure 2A,B). In case of *C albicans*, the median sessile MIC values ranged from 0.125 to 512 mg/L, 0.06 to 1 mg/L, 0.008 to 4 mg/L, 0.03 to 4 mg/L and 0.008 to 4 mg/L for fluconazole, amphotericin B, anidulafungin, caspofungin and micafungin, respectively (Figure 2A). The median biofilm MICs of non-*albicans* species ranged from 0.06 to 1024 mg/L, 0.016 to 1 mg/L, 0.008 to 1024 mg/L, 0.008 to 512 mg/L and 0.008 to 1024 mg/L for fluconazole, amphotericin B, anidulafungin, caspofungin and micafungin, respectively (Figure 2B). *C albicans* sessile MICs to fluconazole and anidulafungin were significantly higher compared with planktonic MIC values ($P < .001$-.05). Similarly, significantly higher biofilm MICs were observed to fluconazole, anidulafungin, caspofungin and micafungin for non-*albicans* species compared with their planktonic MICs ($P < .001$; Figure 2A,B).

We assessed the relationship between biofilm mass or metabolic activity and 30-day mortality, clinical characteristics and underlying conditions (Table 1A and 1). The highest 30-day mortality was observed in *C tropicalis* candidaemia (68%), followed by *C albicans* (62%), *C parapsilosis* (30%), *C krusei* (30%) and *C glabrata* episodes (25%). Significantly higher 30-day mortality was observed in cases of intermediate and high biofilm formers (61%) ($P = .023$; Table 1A). Notably, all *C tropicalis*, *C parapsilosis* and *C glabrata* isolates related to a fatal outcome belonged to intermediate/high category in terms of both biofilm mass and metabolic activity, whereas this ratio was

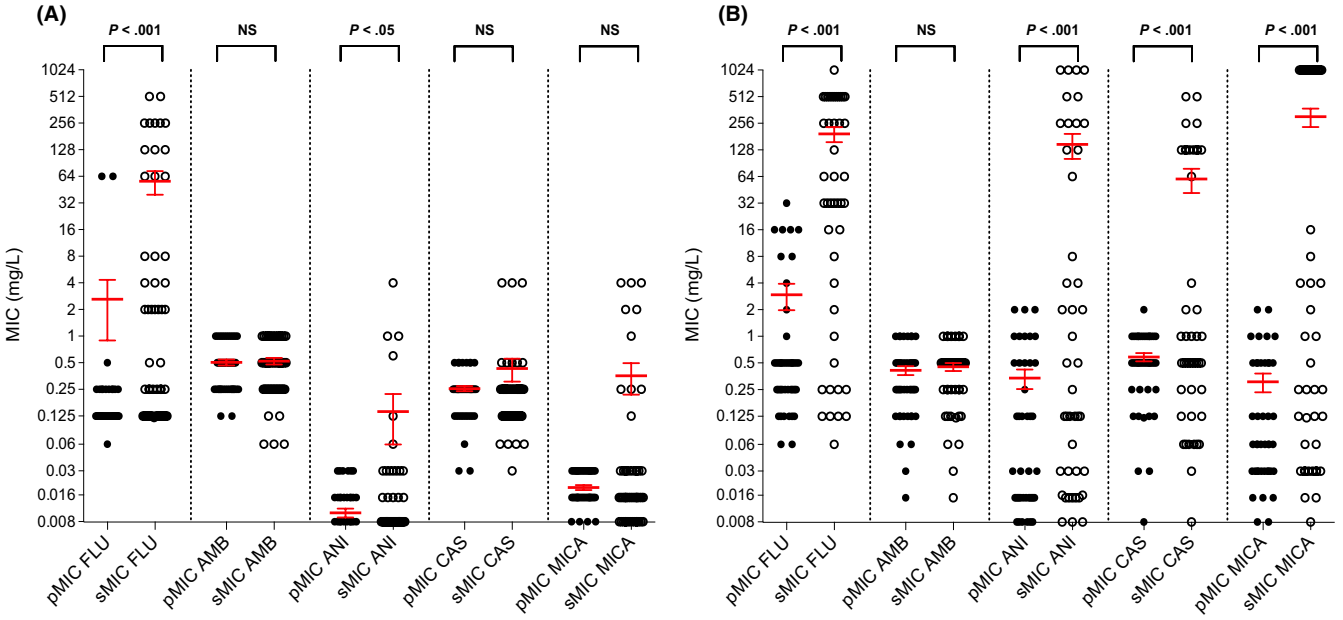

FIGURE 2 Planktonic (pMIC) and sessile MIC (sMIC) distribution of *Candida albicans* (A) and non-*albicans* species (B) biofilms with intermediate and high metabolic activity to fluconazole, amphotericin B, anidulafungin, caspofungin and micafungin. Three replicates were used for each isolate with the median of each represented. The results were considered significant if the *P*-value was < 0.05

85% for *C albicans* isolates. Based on the obtained clinical data, solid malignancy was associated with infections with intermediate/high biofilm-producer *Candida* isolates (*P* = .043; Table 1A). Regarding metabolic activity, the 30-day mortality rate was significantly higher (*P* = .01) in infections caused by biofilm-former *Candida* strains with intermediate/high metabolic activity (62%; Table 1B). In contrast, *Candida* cells with low metabolic activity were associated with a 33% mortality rate (Table 1B). Interestingly, the ratio of bacteraemia was markedly higher (*P* = .015) for *Candida* species with low metabolic activity (53% and 28% for low metabolic activity and intermediate/high metabolic activity, respectively; Table 1B). In multivariate analysis, infection with an isolate producing biofilm with intermediate/high metabolic activity is an independent predictor of mortality (Table 2B), while this association was not demonstrated using crystal violet-based biofilm mass determination (Table 2A).

4 | DISCUSSION

Candidaemia remains a life-threatening hazard to hospitalised patients, especially in intensive care units.[1-8] The highest incidence of candidaemia was reported in Pakistan (21 cases per 100 000) followed by Brazil (14.9/100 000) and Russia (8.29/100 000), while the lowest incidence was observed in Jamaica (5/100 000), Austria (2.1/100 000) and Portugal (2.2/100 00).[25] Based on a recent epidemiological meta-analysis, the incidence of candidaemia in Europe is 3.88 cases per 100 000 corresponding to approximately 79 cases per day.[26] According to a number of studies, patients infected by biofilm-producing isolates have a worse prognosis[8,10,27]; however, contradicting studies also exist.[11,12] This controversy is probably due, at least partly, to variability in the cut-off values used to distinguish low from intermediate and high biofilm producers[12,13] The other possible reason may be the high variability of the examined patient populations.[12,13]

Currently, two in vitro methods (crystal violet and XTT reduction assays) are used to assess the biofilm-forming ability.[14,16] These colorimetric assays are non-invasive and non-destructive compared with alternative methods (eg viable cell counting by flow cytometry).[14,16] However, there is no consensus on the criteria for stratification of biofilm production (as discussed earlier). In this study, we used both of these standardised methods separately to assess simultaneously the impact of biofilm mass as well as metabolic activity on mortality. We applied the method suggested by Rajendran et al (2016) for categorisation of biofilm production, who found a notable difference in 30-day survival between patients infected with low and high biofilm-producing isolates (35% and 41% mortality for low and high biofilm formers, respectively) based on the applied assays, which included XTT assay, crystal violet assay and SYTO-9 assay; in this study, the results of all three assays correlated and their effect was not analysed separately.[10] These observations are consistent with the findings published by Tumbarello et al (2012), who showed 51% mortality in the biofilm-producer group compared with isolates not producing biofilm (32%) based on XTT assay.[8] In contrast, Pongrácz et al (2016) and Muñoz et al (2018) did not confirm biofilm production as a risk factor for mortality.[11,12] In the present study, 30-day mortality caused by *Candida* isolates producing biofilms was significantly higher comparing isolates with low and intermediate/high metabolic activity; in addition, intermediate/high metabolic activity proved to be a significant predictor of mortality in multivariate analysis.

Studies using biofilm mass as predictor of mortality reported similarly versatile results; Muñoz et al (2018) and Shin et al (2002) did not find biofilm development, as determined by a crystal violet or

TABLE 1 Predisposing factors, microbiological characteristics and clinical variables for *Candida* bloodstream infection of patients with candidaemia, including comparison between subgroups according to biofilm mass (A) and metabolic activity of biofilms (B)

	All (n = 127)	Low biofilm formers (n = 30)	Intermediate/high biofilm formers (n = 97)	*P*-value
(A)				
Mean age (range)	61 (1-90)	51 (1-89)	62 (1-90)	.714
Male	75 (59%)	21 (70%)	54 (56%)	.204
Isolated *Candida* species				
C albicans	65 (51%)	15 (50%)	50 (52%)	1.000
C glabrata	4 (3%)	1 (3%)	3 (3%)	1.000
C parapsilosis	23 (18%)	8 (27%)	15 (15%)	.181
C tropicalis	19 (15%)	0 (0%)	19 (20%)	**.007**
C krusei	10 (8%)	4 (13%)	6 (6%)	.245
Other *Candida* spp.	6 (5%)	2 (7%)	4 (4%)	.626
Intensive care unit	100 (79%)	25 (83%)	75 (77%)	.613
Immunosuppression	13 (10%)	3 (10%)	10 (10%)	1.000
Endovascular catheter	99 (78%)	26 (87%)	73 (75%)	.218
Mechanical ventilation	87 (69%)	19 (63%)	68 (70%)	.506
Total parenteral nutrition	68 (54%)	17 (57%)	51 (53%)	.834
Diabetes mellitus	41 (32%)	6 (20%)	35 (36%)	.121
Hypertension	51 (40%)	11 (36%)	40 (41%)	.832
Haematological malignancy	9 (7%)	3 (10%)	6 (6%)	.440
Solid malignancy	19 (15%)	1 (3%)	18 (19%)	**.043**
Abdominal surgery	8 (6%)	3 (10%)	5 (5%)	.392
Bacteraemia	43 (34%)	12 (40%)	31 (32%)	.509
30-day mortality	70 (55%)	11 (37%)	59 (61%)	**.023**
Receipt of systemic antibiotics	91 (72%)	25 (83%)	66 (68%)	.163
Antifungal regimens for treatment				
Fluconazole	102 (80%)	26 (87%)	76 (78%)	.433
Voriconazole	5 (4%)	1 (3%)	4 (4%)	1.000
Amphotericin B	13 (10%)	3 (10%)	10 (10%)	1.000
Caspofungin	15 (12%)	3 (10%)	12 (12%)	1.000
Micafungin	14 (11%)	1 (3%)	13 (13%)	.185
Anidulafungin	3 (2%)	1 (3%)	2 (2%)	.558
Combination antifungal therapy	31 (24%)	7 (23%)	24 (25%)	1.000
(B)				
Mean age (range)	61 (1-90)	63 (1-89)	61 (1-90)	.345
Male	75 (59%)	20 (67%)	55 (57%)	.398
Isolated *Candida* species				
C albicans	65 (51%)	14 (47%)	51 (53%)	.677
C glabrata	4 (3%)	3 (10%)	1 (1%)	**.041**
C parapsilosis	23 (18%)	8 (27%)	15 (15%)	.181
C tropicalis	19 (15%)	0 (0%)	19 (20%)	**.007**
C krusei	10 (8%)	4 (13%)	6 (6%)	.245
Other *Candida* spp.	6 (5%)	1 (3%)	5 (5%)	1.000
Intensive care unit	100 (79%)	25 (83%)	75 (77%)	.613
Immunosuppression	13 (10%)	3 (10%)	10 (10%)	1.000

(Continues)

TABLE 1 (Continued)

	All (n = 127)	Low biofilm formers (n = 30)	Intermediate/high biofilm formers (n = 97)	*P*-value
Endovascular catheter	99 (78%)	25 (83%)	74 (76%)	.614
Mechanical ventilation	87 (69%)	21 (70%)	66 (68%)	1.000
Total parenteral nutrition	68 (54%)	18 (60%)	50 (52%)	.530
Diabetes mellitus	41 (32%)	8 (27%)	33 (34%)	.509
Hypertension	51 (40%)	15 (50%)	36 (37%)	.286
Haematological malignancy	9 (7%)	3 (10%)	6 (6%)	.440
Solid malignancy	19 (15%)	3 (10%)	16 (16%)	.560
Abdominal surgery	8 (6%)	4 (13%)	4 (4%)	.088
Bacteraemia	43 (34%)	16 (53%)	27 (28%)	**.015**
30-day mortality	70 (55%)	10 (33%)	60 (62%)	**.01**
Receipt of systemic antibiotics	91 (72%)	28 (93%)	63 (65%)	**.002**
Antifungal regimens for treatment				
Fluconazole	102 (80%)	26 (87%)	76 (78%)	.433
Voriconazole	5 (4%)	0 (0%)	5 (5%)	.591
Amphotericin B	13 (10%)	2 (7%)	11 (11%)	.731
Caspofungin	15 (12%)	6 (20%)	9 (9%)	.191
Micafungin	14 (11%)	4 (13%)	10 (10%)	.739
Anidulafungin	3 (2%)	0 (0%)	3 (3%)	1.000
Combination antifungal therapy	31 (24%)	9 (30%)	22 (23%)	.468

Note: Fisher's exact test was used to investigate whether any of the risk factors in patients predisposed to infection with biofilm-former isolates and to evaluate whether biofilm production of the isolates had any relationship with 30-d mortality. The results were considered significant if the *P*-value was <.05 (bold).

safranin-related assay, to be associated with increased mortality.[12,28] However, the latter study involved a large proportion of *C parapsilosis* isolates, which are generally associated with low mortality.[28] Rajendran et al (2016) considered biofilm mass as a predictor of mortality, but in their analysis the different markers of biofilm production were not analysed separately.[10] In the present study, mortality was significantly less frequent among patients infected with isolates producing low than with those producing intermediate/high biofilm mass, but biofilm mass was not a predictor of mortality in multivariate analysis.

In our study, we isolated a significantly higher ratio of biofilm-producer strains in patients with solid malignancy. Komor et al (2012) proved that intravenously administered planktonic *Pseudomonas aeruginosa* is enriched in subcutaneous mouse tumours; additionally, they provided evidence that the bacterial cells reside in the tumour tissue within biofilm structures.[29] Pawar et al (2014) showed that *Salmonella enterica* serovar *Typhimurium* can invade solid tumours and produce biofilms similarly to *P aeruginosa*.[30] There is less evidence for an association between fungi and cancer; it was shown that lesions on the oral mucosa have a greater likelihood of undergoing malignant transformation when infected with *Candida* compared with those that are not infected.[31-33] Furthermore, the adherence and biofilm production of *Candida* cells on patient mucosa is higher following radiotherapy, both in terms of number of cells and total biomass production,

TABLE 2 The effect of biofilm production (logarithmised absorbance values with crystal violet (A) or XTT (B)) as well as the different patient characteristics and conditions on patient mortality using multivariate logistic regression models

	Estimate ± Std. Error	*P*-value
(A)		
Intercept	−2.380 ± 0.866	.006
Age	0.038 ± 0.01	.0003
Endovascular catheter	−1.457 ± 0.691	.004
Mechanical ventilation	2.476 ± 0.642	.0001
Total parenteral nutrition	1.203 ± 0.507	.018
Abdominal surgery	−2.098 ± 0.841	.013
Intensive care unit	−1.483 ± 0.722	.039
Receipt of systemic antibiotics	1.131 ± 0.549	.039
(B)		
Intercept	−0.348 ± 0.804	.665
Log XTT absorbance	0.432 ± 0.181	.017
Age	0.031 ± 0.009	.0008
Endovascular catheter	−1.542 ± 0.649	.017
Mechanical ventilation	1.742 ± 0.537	.001
Total parenteral nutrition	1.235 ± 0.503	.014
Abdominal surgery	−1.906 ± 0.914	.037

Note: The results were considered significant if the *P*-value was <.05.

which may be associated with the increased frequency of fungal mucosal infections.[34,35]

We observed candidaemia with bacteraemia in 34% of all cases consistent with the previously described data ranging from 6% to 34.5%.[36] Interestingly, the ratio of candidaemia with concomitant bacteraemia was significantly higher for *Candida* isolates that produced biofilm with low metabolic activity (this was not seen with biofilm mass). Bacteria may release certain compounds that interfere with the metabolism of planktonic and sessile *Candida* cells, interfere with biofilm formation or with fungal quorum sensing. Morales et al (2013) showed that phenazines derived from *P aeruginosa* impair *C albicans* metabolic activity and influence the cellular morphology, cell-cell interactions and biofilm formation.[37] Furthermore, Graham et al (2017) demonstrated that a bacteriocin of *Enterococcus faecalis* potently inhibits hyphal growth.[38]

In conclusion, our analysis confirmed that the ability to form biofilms especially with intermediate/high metabolic activity is related to higher mortality in candidaemia. Furthermore, it highlights that considering biofilm mass and metabolic activity equivalent may lead to conflicting results and these should be analysed and interpreted separately.

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CONFLICT OF INTEREST

László Majoros received conference travel grants from Cidara, MSD, Astellas and Pfizer. All other authors report no conflicts of interest.

AUTHOR CONTRIBUTION

RK conceived the ideas; EV, RK and LM collected the data; FN, ZT, LF and AB performed the biofilm-forming–related tests and susceptibility tests; EV, RK and G. K. analysed the data; and EV, RK, LM and G. K. wrote the manuscript.

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