SHORT THESIS FOR THE DEGREEOF DOCTOR OF PHILOSOPHY (PHD)

The clinicalimportance of *Candida* biofilms and theiralternativetherapeuticapproaches

By EszterVitális, MD

Supervisor: Renátó Kovács, PhD

UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF PHARMACEUTICAL SCIENCES

DEBRECEN, 2022

The clinicalimportance of *Candida* biofilms and theiralternativetherapeuticapproaches

By EszterVitális,MD

Supervisor: Renátó Kovács, PhD

Doctoral School of Pharmaceutical Sciences, University of Debrecen

Head of the Defense Committee:ÁrpádTósaki, PhD, DSc

Reviewers: IstvánVarga, PhD
Marianna Domán, PhD

Members of the Defense Committee: ÁrpádTósaki, PhD, DSc
IstvánVarga, PhD
Marianna Domán, PhD
EnikőFehér, PhD
JuditVáradi, PhD

The PhD Defense will be held on 12 April, 2022. at 1 pm

Live online access will be provided. If you wish to join the discussion, please send an e-mail to the kovacs.renato@med.unideb.hu address until 4 pm at latest on the previous day of the defense (11. April, 2022.). For technical reasons, after the deadline, it will not possible to join the defense.

1. Introduction

According to scientific estimation, about 2,2-3,8 million fungal species exist worldwide, of which roughly 300 can cause human illness. *Candida* species are among the most common human pathogen fungi. About 400 000 *Candida* bloodstream infections are recognized annually globally, and the mortality rate exceeds 40%. The annual incidence of *Candida* bloodstream infections is between 9,5-14,4/100 000 in the United States. In Europe this ranges between 1,4 and 5,7/100 000 and is different in each country.

In the last two decades there is a continuous increase in the incidence of resistant fungal infections due to the uncontrolled use of antifungals in the agriculture, veterinary and human medicine. Additionally, because of global warming and antropogenous effects, some emerging fungal pathogens are recognized in the clinical practice, which are less well known and potentially multiresistant, like *Candida auris*, azole-resistant *Aspergillus* species or *Lomentosporaprolificans*. These newly emerging pathogens pose new challenges to medicine.

Many fungal species can go through morphological changes adapting to environmental conditions, like transformation from yeast to hypha, which is often accompanied by biofilm formation as well. Fungal biofilms play a critical role in the pathogenicity and the antifungal resistance patterns of the species. Unluckily, currently we have very few antifungal drugs that have a potent anti-biofilm activity, therefore new types of antifungal therapeutic approaches are considered the way to the solution by clinicians. The most promising antifungal agents are in phase III. stage of development. These include ibrexafungerp, rezafungin, a new itraconazole formulation with high bioavailability, and a new azole molecule, named VT-1161. Alternative strategies that were examined in recent times include: high dose therapy with classic antifungal agents, antifungal lock therapy and combination-based strategies. According to *in vitro* and *in vivo* data, echinocandins and amphotericin B are the most promising basic agents for combinational approaches, and molecules that confuse quorum-sensing can also be the basis for innovative therapeutic options. Used in supraphysiological concentrations, quorum-sensing molecules can interact with intercellular communication within the biofilm, which leads to the disruption of the solid structure of the matrix. Hopefully, these new innovative, combined therapies can be effective against the newly emerging, more resistant *Candida* species, like *C. auris*. The results of our study can help to introduce new therapeutic strategies to better fight the present and future multiresistant fungal pathogens.

2. Objectives

We were curious about the biofilm-producing properties (biofilm mass and metabolic activity) of the *Candida* species isolated from blood cultures in Debrecen University. We were searching for any association between the presence of biofilm and mortality or comorbidities. In the second part of the experiments, we were studying the effect of farnesol exposition on *C. auris* clinical isolates, for the purpose of suggesting new therapy against this potentially multiresistant nosocomial pathogen.

We set us the following targets during our studies:

- Studying biofilm-producing properties of *Candida* isolates from blood cultures and investigating planktonic and sessile resistance profiles.
- Establishing relationship between biofilm producing ability and mortality or any predisposing factors/comorbidities.
- Studying *in vitro* effect of farnesol on *C. auris* planktonic cells and one-day-old biofilms.
- Studying *in vitro* interaction between farnesol and azoles against *C. auris*.
- Studying *in vivo* effect of farnesol in neutropenic mice infected with *C. auris*.

3. Materials and methods

3.1. Inclusion criteria for clinical isolates used for studying biofilm producing properties

A total of 127 *Candida* isolates have been investigated, that were collected between January 2013 and December 2018 from the clinics of Debrecen University (1667 beds) from 127 independent candidaemia episodes. Cases were considered candidaemia when at least one *Candida* species was cultured from at least one haemoculture of a patient. If *Candida* was cultured more than once from the same patient, we considered the two events two different episodes of candidaemia if they occurred at least 30 days apart. Patients, whose haemocultures grew different *Candida* species at the same time, were excluded. We collected data about the patients' demographics, comorbidities, and antimicrobial treatment by reviewing the charts in the electronic medical system. We followed outcome of candidaemia for 30 days from the first positive culture or until the patient's death.

Fisher-exact test was used for detecting if any predisposing factor exists for being infected with biofilm-producing strain, or if the presence of a biofilm-producing strain is in association with 30-day mortality. We analyzed the effect of biofilm producing ability of the strain and the characteristics of the patients and the other conditions on mortality with multivariate regression model. Results were considered significant if*p* value was smaller than 0,05.
The study was authorized by the Regional and Institutional Ethics Committee, University of Debrecen Clinical Center on the following number: 5190-2019.

3.1.1. Origin of *Candida auris* isolates

In our *C. auris* studies we used three clinical isolates (isolate number 10, 12, 27), which were sent from the National Microbiological Reference Laboratory of the United Kingdom. As a comparative control we used the SC5314 *C. albicans* referential isolate. It is important to emphasize, that all examined *C. auris* isolates showed non-aggregating phenotype, which usually demonstrate pathogenicitysimilar to that of *C. albicans*.

3.2. Studying biofilm production of the clinical isolates from candidaemia

Isolates were subcultured on Sabouraud dextrose agar (SDA) 48 hours before the experiment. 24 hours before the biofilm-experiments the isolates were spread on SDA. The cultures were incubated at 37 °C, for 24 hours, then suspended into 25-30 ml physiological saline with a sterile cotton swab. Then fungal cells were washed three times, during which the suspensions were centrifuged at 3000 g for 5 minutes, followed by suction of the supernatant and suspension of the fungal cells in 25 physiological saline solution again. $1x10^6$ Colony Forming Unit (CFU)/ml fungal suspensions were made in RPMI-1640 from the different isolates. Thereafter 100 µl was pipetted into dedicated wells of polystyrene flat-bottom 96-well microtiter plate (TPP, Trasadingen, Switzerland). The 12th column of the plate served as negative control, containing only 100 µl of RPMI-1640. Finally plates were incubated statically for 24 hours at 37 °C.

For measuring the biomass, after removing the content of the wells and washing them 3 times with 200 µl physiological saline, 125 µl of 0,1% crystal violet solution was pipetted into the wells, then the plate was incubated for 15 minutes on room temperature. Then the crystal violet was suctioned, and the biofilms washed three times with 200 µl physiological saline. As a final step of the protocol, 125 µl of 33% acetic acid solution was added to the wells of the plate to dissolve the crystal violet bound to the biofilm. After 15 minutes of room-temperature incubation, 100 µl of the supernatant was pipetted into a sterile microtiter plate. Absorbance was read by spectrophotometry at 540 nm. As negative control 100 µl of 33% acetic acid was used.

Metabolic activity of biofilms was examined by using XXT [2,3–bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide]-assay (VWR, Debrecen, Hungary): after washing the biofilms three times with physiological saline, 100 µl XTT/menadion solution (0,5 g/l XTT, plus 1 µM menadion) was added to the wells of the plate, which was then incubated statically in dark for 2 hours at 37 °C. 80 µl of supernatant was used for spectrophotometry at 492 nm.

As the results of the crystal violet- and the XTT-assay do not necessarily correlate with each other, and, as a consequence, they should be considered as two different characteristics of the biofilms, we categorized the isolates according to their biofilm mass and metabolic activity as biofilms showing low, intermediate or high biofilm mass and low, intermediate or high metabolic activity according to a recently published paper. Isolates belonging to the first quartile (Q1) were considered low biofilm producers, the ones producing more biofilm mass than the third quartile (Q3) were considered high biofilm mass producers, and the ones

between these two categories (Q2) were considered intermediate biofilm producers. The same principles were followed when categorizing the isolates according to their biofilms' metabolic activity. To be easily comparable with other similar studies, isolates were grouped into low versus intermediate/high biofilm mass and low versus intermediate/high metabolic activity.

Analysis of the biofilm mass and metabolic activity of different *Candida* isolates was done by Kruskal-Wallis-test completed with Dunn's multiple comparison test.

3.3. Susceptibility testing of the planktonic cells

Evaluation of MIC values of *Candida* isolates against fluconazole (Sigma, Budapest, Hungary), amphotericin B (Sigma, Budapest, Hungary), caspofungin, micafungin and anidulafungin (Molcan, Toronto, Canada) were done by standard microdilution assay according to the M27-A3 protocol of Clinical Laboratory Standards Institute (CLSI). As medium, L-glutamine enriched, hydrogen-carbonate-free RPMI-1640-et was used (pH=7,0), with the adding of MOPS [3-(N-morpholino) propane- sulfonate) Sigma] puffer) (Clinical and Laboratory Standards Institute 2008). The examined drug-concentrations were 0,03-32 mg/L, 0,016-8 mg/L and 0,008-4 mg/L for fluconazole, amphotericin B and the three echinocandins. All isolates were tested in triplicate and median values were used for further evaluation. *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 isolates were used as control in all experiments. Reading of the planktonic MIC values was done after incubation at 37 ^{o}C for 24 hours. Regarding fluconazole and the tested echinocandins, partial growth reduction (at least 50% decrease in turbidity as compared to the control) was considered MIC, and for amphotericin B, MIC was the lowest concentration, where 100% growth reduction was seen as compared to the control. We analyzed differences between planktonic and sessile susceptibilities with Wilcoxon matched-pairs test.

MIC evaluation of *C. auris* isolates against fluconazole, itraconazole, voriconazole, posaconazole, isavuconazole and farnesol (all from Sigma, Budapest, Hungary) were done, as earlier, with standard microdilution assay according to M27-A3 protocol approved by Clinical Laboratory Standards Institute (CLSI). Drug concentrations were between 0,5 and 32 mg/L, 0,008 and 0,5 mg/L, and 1,17 and 300 μM mg/L for fluconazole, the other examined azoles and farnesol. In all cases, MIC was the lowest concentration, where we first observed 50% growth reduction as compared to the control.

3.4. Antifungal susceptibility of biofilms

Anti-biofilm activity of the examined antifungals was measured by XTT-assay. Concentrations for anti-biofilm MIC evaluation ranged between 0,06-512 mg/l, 0,015-8 mg/l and 0,008-4 mg/l for fluconazole, amphotericin B and the examined echinocandins. For *C. parapsilosis*, echinocandin-concentrations were between 2 and 1024 mg/L. For determining sessile MIC, biofilms were washed three times with 200 μL sterile physiological saline. XTT assay was done as described previously. MIC value was the concentration, where we first observed 50% reduction in metabolic activity as compared to the untreated control biofilm. Percental change of metabolic activity caused by drug-treatment was calculated as follows: $(A_{well} - A_{negative\ control})/(A_{positive\ control} - A_{negative\ control})$, where *A* is the measured absorbance. All isolates were tested in a triplicate, and median values were used for further analysis. In all experiments with biofilms, *C albicans* SC5314 reference strain was used as quality control. Analysis of the differences between MIC values against the given antifungals was done by Wilcoxon matched-pairs tests.

Activity of triazoles and farnesol against one-day-old *C. auris* biofilms was measured by XTT-assay as described above. Examined concentrations were between 8 and 512 mg/L, 0,5 and 32 mg/L, 0,125 and 8 mg/L, and 1,17 and 300 μM for fluconazole, voriconazole/itraconazole, posaconazole/isavuconazole and farnesol.

3.5. Effect of farnesol on planktonic growth of *Candida auris*cells

Effect of pre-exposition and continuous farnesol treatment on fungal growth was evaluated in the following experimental models:(i) effect of different farnesol-concentrations against planktonic cells, (ii) effect of different farnesol-concentrations against planktonic cells pre-exposed with farnesol (75 μM) for 24. 75 μM farnesol was chosen as pre-exposition concentration, because this is approximately the double of physiological farnesol-production of *C. albicans*, therefore it can be considered supraphysiological, but it doesn't show toxicity yet. Examined farnesol concentrations were 10, 50, 100 and 300 μM in all experiments.

Number of planktonic cells was evaluated by time-kill experiments. Samples (100 μL) were taken at 0, 2, 4, 6, 8, 10, 12 and 24 hours. Ten-fold dilution was done from each sample, and

4 × 30 µL was pipetted onto SDA, then incubated at 37°C for 48 hours, thereafter the grown colonies counted. All isolates were measured in a triplicate, and mean values were presented. For analyzing the effect of farnesol on the number of living cells, univariate variance analysis complemented with Dunnett-test was used. Results were considered significant, if*p* was < 0,05.

3.6. Effect of farnesol on *Candida auris*biofilms

Effect of farnesol pre-exposition and continuous farnesol treatment on *C. auris* and *C. albicans* biofilms was examined in three experimental protocols: (i) continuous farnesol treatment during the 24 hours of biofilm formation, (ii) biofilm-producing ability of cells pre-exposed to farnesol (75 µM) before the biofilm formation, then farnesol treatment during biofilm formation for 24 hours with given farnesol concentrations, (iii) effect of different farnesol-concentrations on one-day-old biofilms. Examined farnesol concentrations were 10, 50, 100 and 300 µM in all experiments. Metabolic activity of sessile cells was evaluated at 0, 2, 4, 6, 8, 10, 12 and 24 hours by XTT-assay. All isolates were measured in a triplicate and mean values were used for further analysis. For analyzing the effect of different farnesol-concentrations on the changes in metabolic activity in the given time-points, univariate variance analysis completed with Dunnett-test was used. Differences between the treated and untreated cells were considered significant, if *p* value was less, than 0,05.

3.7. Examination of *in vitro* interactions between azoles and farnesol

For the examination of *in vitro* interactions between the given drugs, two-dimensional „checkerboard" microdilution assay was used for both planktonic and sessile *C. auris* cells. In the case of *C. albicans* we did not examine the planktonic cells, because high-degree of azole-susceptibility was seen in all cases.

Row „A" of the plates contained the different farnesol concentrations alone, while the 10th column contained the different azole-concentrations on their own during "checkerboard" microdilution assay. In the remaining wells of the plate, different combinations of the two drugs were pipetted. 11th column of the plate served as untreated positive control, while 12th row as so called medium-control. For defining the nature of the interaction, fractional inhibitory concentration index (FICI) was used. FICI was calculated as follows: $\Sigma FIC = FIC_A +$

FIC_B = MIC_A in combination / MIC_A alone + MIC_B in combination / MIC_B alone, where MIC_A and MIC_B alone are MIC values of drug A and B alone, while MIC_A and MIC_B in combination is the MIC value for the drugs in isoeffective concentration. In the case of all isoeffective combinations, FIC value was calculated, and the lowest FIC value was considered FIC index (FICI), which was interpreted as follows: interaction was considered synergistic, if FICI was ≤0,5, indifferent if 0,5<FICI≤4, and antagonistic if FICI was >4 .

3.8.*In vivo* effect of farnesol in neutropenic mouse model

BALB/c neutropenic female mice (21-23g) were used to investigate the effect of farnesol pre-exposition (75 μM) and daily farnesol treatment (75 μM) against the virulence of a representative *C. auris* isolate and *C. albicans* SC5314 strain. Animals were kept according to the guidelines about laboratory animal care. All experiments were accepted by the Animal Welfare Committee of University of Debrecen (Debrecen, Hungary) (permission number: 12/2014 DEMÁB). For sustained immunosuppression, 150 mg/kg cyclophosphamide was administered intraperitoneally 4 days before the infection, then 100 mg/kg cyclophosphamide 1 day before the infection, again 100 mg/kg 2 days and 5 days after the infection. According to our previous experiments, mice were infected intravenously through the lateral tail vein; the infective doses were 1×10^7 CFU/mouse and 8×10^3 CFU/mouse in 0,2 ml volume for *C. auris* and *C. albicans,* respectively. Mice were divided into 4 groups (10 mice per group); (i) untreated control mice; (ii) infection with cells pre-exposed to farnesol (75 μM) for 24 hours; (iii) no farnesol pre-exposure before infection, but daily 75 μM farnesol was started 24 hours after inoculation; (iv) farnesol pre-exposition for 24 hours (75 μM) on yeast cells before infection, then daily 75 μM farnesol started 24 hours after inoculation.

Treatment was administered intraperitoneally in 0,5 ml physiological saline. Control mice got only 0,5 ml saline intraperitoneally. 6 days after the infection, mice were euthanized and kidneys removed, measured and aseptically homogenized. Fungal burden of the kidneys was determined by quantitative culture. Results were analyzed by Kruskal-Wallis test with Dunn's multiple comparison test. Result was considered significant if p value was less than 0,05.

Kidneys of treated and untreated mice were also investigated histologically. Histological examination and stain was done on mouse-kidney tissue fixed in formalin, embedded in paraffin. From the paraffin-blocks, 4 μm-thick slices were cut and PAS-stain done.

4. Results

4.1. Results of epidemiological examinations of biofilm-production

Major part (59%) of the examined clinical isolates originated from male patients, and mean age was 61 years. 79% of the patients were treated in the ICU. Regarding the prevalence of different *Candida* species, *C. albicans* was the most common, causing 51% (65/127) of the investigated candidaemia episodes, followed by *C. parapsilosis* (23/127; 18%), *C. tropicalis* (19/127; 15%), *C. krusei* (10/127; 8%), *C. glabrata* (4/127; 3%) and other, less common species (6/127; 5%). *C. lypolitica*, *C. catenulata*, *C. guilliermondii*, *C. dubliniensis*, *C. inconspicua* and *C. orthopsilosis* caused one candidaemia episode each.

Biofilm mass and metabolic activity of sessile cells was quite heterogeneous, independent from the examined species. However, *C. tropicalis* isolates had a significantly greater biomass than other *Candida* species ($p<$ 0,001-0,05); furthermore, their metabolic activity was significantly higher than that of *C. glabrata* and *C. krusei* (p <0,01-0,05).

For the measurement of the biofilm mass formed by different *Candida* isolates, crystal violet assay was used. Isolates were considered low (Q1), intermediate (Q2) or high (Q3) biofilm-producers, if their absorbance was lower than 0,01 ($OD_{540\ nm}$), between 0,01 and 0,276 ($OD_{540\ nm}$) or more than 0,276 ($OD_{540\ nm}$) respectively. 15, 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 categorized low, intermediate, and high biofilm-producers, respectively. Regarding the less common species, the examined C. *catenulata* and *C. inconspicua* isolates were low, whereas *C. lypolitica*, *C. guilliermondii*, *C. dubliniensis* and *C. orthopsilosis* isolates were intermediate biofilm-producers according to their absorbance values.

Different sessile *Candida* isolates were also categorized – similarly to crystal violet-based experiments - based on their metabolic activity, measured by XTT-assay. Low (Q1), intermediate (Q2) and high (Q3) metabolic activity was defined as absorbance less than 0,019 ($OD_{492\ nm}$), between 0,019 and 0,149 ($OD_{492\ nm}$) or more than 0,149-es ($OD_{492\ nm}$), respectively. 14, 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* fell into the low, intermediate or high metabolic activity categories, respectively. The examined *C. catenulata* had low metabolic activity, while the *C. inconspicua*, *C. lypolitica*, *C. guilliermondii*, *C. dubliniensis* and *C. orthopsilosis* isolates had intermediate metabolic activity.

Planktonic MIC values of the isolates were between 0,125 and >32 mg/l; 0,06 and 1 mg/l; 0,008 and 2 mg/l; 0,03 and 1 mg/l; and between 0,008 and 2 mg/l against fluconazole, amphotericin B, anidulafungin, caspofungin and micafungin. According to current CLSI breakpoints, 3 *C. albicans* and all *C. krusei* showed fluconazole resistance. Moreover, one of the *C. inconspicua* isolates showed decreased susceptibility against fluconazole (MIC = 8 mg/L). All fluconazole-resistant *C. albicans* strains, 8 *C. krusei* isolates and one *C. inconspicua* had low biofilm mass and low metabolic activity. As for echinocandin resistance, 19 *C. albicans*, 6 *C. tropicalis*, 6 *C. krusei* and 3 *C. glabrata* isolates showed reduced susceptibility against caspofungin. No echinocandin resistant strains were found.

Sessile MIC values of *C. albicans* were between 0,125 and 512 mg/l; 0,06 and 1 mg/l; 0,008 and 4 mg/l; 0,03 and 4 mg/l; and between 0,008 and 4 mg/l against fluconazole, amphotericin B, anidulafungin, caspofungin and micafungin, respectively. Median values of biofilm MIC values of non-*albicans* species fell between 0,06 and 1024 mg/l; 0,016 and 1 mg/l; 0,008 and 1024 mg/l; 0,008 and 512 mg/l; and between 0,008 and 1024 mg/l against fluconazole, amphotericin B, anidulafungin, caspofungin and micafungin, respectively. Sessile MIC values of *C. albicans* against fluconazole and anidulafungin were significantly higher than planktonic ones ($p<$ 0,001-0,05). Similarly, sessile MIC values of non-*albicans* species against fluconazole, anidulafungin, caspofungin and micafungin were significantly higher than planktonic ones ($p < 0,001$).

Relationship between biofilm mass, metabolic activity and comorbidity of patients was also investigated. Highest mortality was seen in *C. tropicalis* candidaemia (68%) followed by *C. albicans* (62%), *C. parapsilosis* (30%), *C. krusei* (30%) and *C. glabrata* (25%). 30-day mortality was significantly higher in the case of intermediate or high biofilm mass (61%) (p = 0,023). Remarkably, all *C. tropicalis*, *C. parapsilosis* and *C. glabrata* isolates with lethal outcome fell into the intermediate/high category in terms of biofilm mass and metabolic activity as well, while this rate was 85% in case of *C. albicans* isolates. On the basis of the results, a relationship could be established between solid malignancy and infection with intermediate/high biofilm producing strains (p = 0,043). 30-day mortality was significantly higher (p = 0,01) in infections caused by strains showing intermediate/high metabolic activity (62%). As an opposite, isolates with low metabolic activity were associated with a mortality rate of 33%. Interestingly, bacteraemia occurred significantly more often (p = 0,015) in infections caused by strains with low metabolic activity (bacteraemia was occurring in 53% in the low metabolic activity group vs. 28% in the intermediate/high metabolic activity group).

Regression analysis proved, that infection with an isolate with intermediate/high metabolic activity is an independent risk factor of mortality, while this association was not demonstrable on the data obtained by crystal-violet-based biofilm mass experiments.

4.2. Effect of farnesol on *Candida albicans* and *Candida auris* planktonic cells

Farnesol significantly blocked growth of *C. auris* cells in the first 12 hours in concentrations between 50 and 300 μM in both models, namely when using farnesol pre-exposed or non-exposed cells ($p<$ 0,001-0,05). After 24 hours, 100 and 300 μM farnesol significantly suppressed the number of viable cells in both models, as compared to the non-treated cells. ($p<$ 0,01-0,001). Surprisingly, until 24 hours, nor the farnesol pre-exposed, neither the normal, non-exposed cells did not show significant growth reduction in the case of *C. albicans* ($p>$ 0,05).

4.3. Effect of farnesol on *Candida albicans* and *Candida auris* biofilm-production and on one-day old biofilms

4.3.1. Effect of different farnesol-concentrations on biofilm producing ability

All investigated farnesol concentrations decreased the metabolic activity of *C. auris* cells in the first 8 hours, as compared to the control cells ($p<$ 0,001-0,05); while, after 24 hour exposition, statistically similar metabolic activity was measured as in the control cells ($p>$ 0,05). In opposite, all investigated farnesol concentrations decreased the metabolic activity of *C. albicans* cells during the investigated 24 hours.

4.3.2. Biofilm-producing ability in cells pre-exposed to farnesol (75 μM) before biofilm production

Interestingly, in the metabolic activity of *C. auris* cells, statistically significant difference could be shown only at 24 hours, and only at 50 and 300 μM concentrations. In the case of C. *albicans*, statistically significant differences in metabolic activity, with farnesol-concentrations between 50 and 300 μM could be detected from 8 hours, but metabolic activity of cells treated with different concentrations did not differ at 24 anymore.

4.3.3. Effect of different farnesol concentrations against one-day old biofilms

Regarding metabolic activity, 300 μM farnesol treatment had a significant anti-biofilm effect between 2 and 24 hours in *C. auris*, as compared to control. Contrarily and interestingly, low farnesol concentrations (10-50 μM) increased the metabolic activity of *C. albicans* biofilms in the first 4 hours. After 24 hours, however, different farnesol treatments did not differ statistically in the case of *C. albicans*.

4.4. Susceptibility profile of planktonic and sessile *Candida albicans* and *Candida auris*cells

For *C. auris* isolates, planktonic MIC values ranged between 4 and >32 mg/l, 0,03 and 0,06 mg/l, 0,008 and 0,015 mg/l, 0,015 and 0,03 mg/l, and between 0,008 and 0,015 mg/l against fluconazole, voriconazole, isavuconazole, itraconazole and posaconazole. MIC of isolate number 10 was higher than the MIC breakpoint for fluconazole (>32 mg/l), while the other two strains were susceptible for fluconazole. (Centers for Disease Control and Prevention, 2020). Median value of MICs of planktonic *C. albicans* SC5314 reference strains was 0,125 mg/l, 0,015 mg/l, 0,015 mg/l, 0,125 mg/l and 0,008 mg/l against fluconazole, voriconazole, isavuconazole, itraconazole and posaconazole. In case of *C. auris* isolates, sessile MICs ranged >512 mg/l, 64 mg/l, 16-32 mg/l, 16 mg/l and 4-8 mg/l for fluconazole, voriconazole, itraconazole, posaconazole and isavuconazole, respectively.

4.5. Analysis of the interactions between triazoles and farnesol against *Candida auris* and *Candidaalbicans* biofilms

In the case of *C. auris* planktonic cells, only indifferent interactions could be detected. Results of triazole-farnesol interactions against one-day-old biofilms, determined by FICI were as follows: antagonism was never seen; synergism between triazoles and farnesol was detectable in all three *C. auris* sessile isolates (FICI between 0,038 and 0,375). Interaction pattern against *C. albicans* SC5314 strain was very similar to *C. auris*; indifferent azole-farnesol interactions were only seen in the case of fluconazole, however, the calculated FICI was very close to the breakpoint of synergism.

4.6. Investigation of *in vivo* effect of farnesol against *Candida albicans* and *Candida auris*

75 μM farnesol treatment significantly decreased the number of living fungal cells cultured from the kidneys, especially, when farnesol pre-exposed *C. auris* cells were used as inoculum. In the case of *C. albicans* all experimental models resulted in statistically indifferent living cell numbers as compared to the control. Histological results were in accordance with the results of the quantitative culture. Infection with *C. auris* resulted in numerous separate yeast cells and budding yeast cells in the untreated control mice. However, inoculation of farnesol pre-exposed *C. auris* cells into mice resulted in numerous fungal cell aggregates in the kidneys; daily farnesol treatment decreased the number of these changes. Both farnesol pre-exposure and daily treatment resulted in numerous, broad pathological changes in the kidney tissues in the case of *C. albicans* infection, where separate and budding yeast cells, pseudohyphae and hyphae were detectable.

5. Discussion

Candidaemia is still the most dangerous form of invasive candidiasis, with the highest mortality rates, and it is a real threat to the hospitalized patients, mostly in the intensive care units. In recent epidemiological papers, prominently high incidence was reported from Pakistan (21 cases/100 000), from Brazil (14,9/100 000), and Russia (8,29/100 000). Lower mean incidence rates were reported from Jamaica (5/100 000), Austria (2,1/100 000) and Portugal (2,2/100 00). According to a meta-analysis conveyed throughout whole Europe, the mean incidence of candidaemia on the „old continent" is 3,88 cases per 100 000 patients, which means 79 cases daily. As for first reading, it does not seem to be high, but to these relatively few cases high mortality rates are associated (40-50%). According to many studies, the prognosis of patients infected with biofilm producing strains is significantly worse; but it is worth noting, that some contradicting studies also exist, where discrepancy is usually based on different inclusion criteria, and different breakpoints for biofilm production. Another possible cause is the high degree of variability in patient populations.

Currently, two *in vitro* assays (crystal violet-assay and XTT-assay) are used widely to assess biofilm production, due to their relatively cheap and easy methods without destruction of the cells, like in other experimental approaches (e.g. counting the viable cells by flow cytometry). A severe limitation of epidemiological or basic studies on biofilm production is the absence of widely accepted consensus about criteria for biofilm production itself.

In our current study, both widely prevalent assays were used to assess the effect of biofilm mass and metabolic activity on mortality of candidaemia. As there is no current consensus on the breakpoints of biofilm production in a given*Candida* isolate, we used the method described earlier to determine whether an isolate is biofilm producer or not. Significant difference between 30-day survival of patients infected with low or high biofilm producer strains according to XTT-assay, crystal violet-assay and SYTO-9-stain was found in recent papers (35% and 41% mortality rate for low or high amount of biofilm production). All three tests showed significant correlation between biofilm-production and outcome of candidaemia. These results are in correspondence with our observations, where mortality was found to be 61% in candidaemia cases caused by biofilm producing strains determined by XTT-assay, versus 32% in the non-biofilm-producing group. In opposite, some authors did not confirm the biofilm production as an independent risk factor for mortality in candidaemia.

According to our results, 30-day mortality in candidaemia caused by isolates with intermediate/high metabolic activity was significantly higher than that in infections caused by

isolates with low metabolic activity. It is also important to emphasize that intermediate/high metabolic activity was shown to be an independent risk factor of mortality by multivariate regression analysis.

Earlier no relationship could be established between mortality and biofilm mass measured by crystal violet or safranin. It is noteworthy that in one of these studies, *C. parapsilosis* was overrepresented, and infections caused by this species lead to death less frequently due to their low virulence, and this fact could bias the results of the study. In the experiment cited earlier, the thickness and robustness of the biofilm was found to be a significant predictor of death. In our study, mortality among patients infected with a strain producing few biofilm, mortality was significantly lower than among patients infected with isolates showing greater biofilm mass production. Though biofilm mass measured by crystal violet assay has not been shown to be an independent risk factor of death according to multivariate regression analysis.

When examining the predisposing factors in our study, isolates producing high amount of biofilm were found more frequently in patients with solid malignancy. It has been proven recently, that planktonic *Pseudomonas aeruginosa* inoculated intravenously was able to accumulate in subcutaneous tumors of mice; moreover evidence was also presented, that bacterial cells show sessile or biofilm phenotype in cancer tissues. Other authors have shown that *Salmonella enterica serovar Typhimurium* is able to enter solid tumors and form biofilms inside. In recent studies, much less evidence can be found about the association between biofilm producing ability of fungi and cancer. However, oral mucosal lesions have more probability to progrediate into malignancy if *Candida* is also present simultaneously. Moreover, adhesion and biofilm production of *Candida* cells is increased after radiotherapy on the mucosal surface as determined by either living cell number either extent of biofilm, which can be associated with the increased rate of mucosal fungal infections in this patient population.

In our study, co-incidence of bacteraemia and candidaemia was observed in 34% of all candidaemia cases, which is in accordance with previously described rates of 6% to 34,5%. Interestingly, rates of candidaemia and concomitant bacteraemia were higher in cases of *Candida* isolates with low metabolic activity biofilms. Bacteria can produce some molecules which may interfere with the metabolism of planktonic and sessile *Candida* cells, with biofilm production or interfungal quorum-sensing. Recently it has been shown that fenasinesderived from *P. aeruginosa* impair metabolic activity of *C. albicans*, and influence cellular morphologyand biofilm production. Others observed that one of the bacteriocins of *Enterococcus faecalis* block bacterial growth, which also influences metabolic activity,

negatively. Our current analysis thus verified that presence of biofilms, mostly the ones with intermediate/high metabolic activity is in association with higher mortality in case of candidaemia patients. It also highlights that considering biofilm mass and metabolic activity interchangeable, can lead to conflicting conclusions, thus these two factors should be analyzed and interpreted independently in similar studies.

Although it has a more than 10-year-old clinical presence, *C. auris* is still a great challenge in clinical practice. This potentially multiresistant species can cause candidaemia with high frequency, which is characterized with high mortality, especially in intensive care units. Eradication of this pathogen is difficult due to its biofilm production;thus novel therapeutic approaches are needed in the future. The number of available systemic antifungal drugs is strongly limited; moreover, discovery of new antifungals is slow and challenging, especially against newly emerging difficult-to-treat pathogens such as *C. auris*. As alternative therapeutic strategy against C. *auris*, combination-based approaches are advised. Flucitozin with amphotericin B or micafungin can be efficacious against *C. auris* infections. Moreover, a synergistic interaction has been described between micafungin and voriconazole, similarly to isavuconazole and caspofungin. In the recent years more and more are known about alternative strategies based on interference with quorum-sensing, in which the antimicrobial properties of farnesol or tyrosol have been investigated. Many *in vitro* and *in vivo* experiments have been conducted to discover antimicrobial effects of farnesol which suggested that this molecule could serve as monotherapeutic agent or as combination partner for traditional antifungal drugs. In physiological concentrations farnesol has diverse actions, from which the most prominent is the effect on *C. albicans* morphology. It is noteworthy that farnesol not only has an impact on *C. albicans*, but also on other non-albicans species and molds – especially in supraphysiological concentrations.

In a recent paper we described that farnesol has potential antifungal activity against *C. auris* biofilms, although the physiological background of this phenomenon is not yet clarified. According to our results, farnesol did not have an effect on the growth of planktonic *C. albicans* cells; however, it showed significant growth-reduction impact on *C. auris*. Regarding sessile cells, farnesol blocked the metabolic activity of one-day-old biofilms in the first 24 hours, which was not seen in the case of *C. albicans*.

Several epidemiological studies have reported catheter-associated infections caused by *C. auris*, which is due to its previously described biofilm-producing properties. Recent investigations have found high prevalence of catheter-associated infections caused by *C.*

auris: 11-92% of all invasive *C. auris* infections were related to the presence of a central venous catheter. Although significantly higher resistance rates are seen in sessile communities against widely used antifungals, as compared to planktonic resistance patterns, the effect of antifungals can be increased by such adjuvants as farnesol. A clear synergism has been shown between triazoles and farnesol against *C. auris* biofilms, similarly to the combination of echinocandins and farnesol. Probably farnesol interferes with expression of genes involved in the synthesis of ergosterol, which could explain synergism between triazoles and farnesol.

Although *in vitro* effect of farnesol is well described, especially against *C. albicans*, it's*in vivo* role is still inconsistent and raises several questions, regarding its clinical usefulness. It has been shown that exogenous farnesol (20 mM/mouse) can increase pathogenicity of *C. albicans*, also increasing mortality in systemic candidiasis mouse model. In contrast, other authors observed a protective effect of farnesol (in a dose of 9 μM/mouse) in *C. albicans*-associated oropharyngeal candidiasis. Although our colleagues showed that farnesol alone is not protective in vulvovaginitis (150-300 μM/mouse), it improves the activity of fluconazole against fluconazole-resistant *C. albicans* isolates. Recently, scientists created nanoparticles containing miconazole and farnesol, where farnesol concentration was ≥240 μM.

In our current study, daily farnesol treatment suppressed the number of viable *C. auris* cells in the kidneys of mice, independently from the former farnesol-exposition of the inoculum. Furthermore, in the case of inoculums pre-exposed to farnesol, the decrease in the number of viable yeast cells was statistically significant, which is in accordance with our *in vitro* growth results. The observed antifungal activity can be explained by the previously measured elevated levels of reactive oxygen derivates, which were not observed in similar experiments with *C. albicans*. Furthermore, lipophilic properties of farnesol enable its integration into the fungal cell wall, thus influencing membrane fluidity and integrity. According to a recent study, farnesol has an impact on cell-polarization and membrane permeability, which can also explain the antifungal activity described in our study. Noteworthy, inoculation of farnesol pre-exposed fungal cells without daily farnesol treatment led to a more serious infection and more severe kidney damage in mice. 24-hour farnesol pre-exposition without consecutive farnesol treatment might have an influence on the expression of virulence factor genes and on the membrane characteristics similarly to fluconazole pre-exposition, which can explain the increase in virulence in this setting.

Investigation of the relationship between mortality in candidaemia and biofilm production was major part of my work, and we could show that metabolic activity of biofilms has a

significant impact on 30-day mortality in candidaemia. Physiological background of the antifungal effect of farnesol has also been in focus. Based on our results, new perspectives are in sight for the treatment of infections caused by *C. auris*, and the data obtained from the investigation of isolates from candidaemia underline the importance of biofilm production, which can determine the outcome in candidaemia patients.

6. Summary

Candidaemia is a common life-threatening disease among hospitalized patients. In addition, several newly emerged *Candida* species such as *Candida auris* contribute to worrisome mortality and epidemiology trends of candidaemia. The spreading of multidrug-resistant *C. auris* is considered as an emerging global threat. Biofilm formation is an important virulence factor for several *Candida* species; however, the effect of the biofilm-forming ability of *Candida* strains on the clinical outcome remains controversial. Moreover, the number of effective therapeutic regimens against biofilms and/or newly emerged *C. auris* planktonic cells and biofilms is strongly limited; therefore, development of novel strategies is urgently needed. The first aim of my PhD project was to determine the impact of biofilms, specifically focusing on biofilm mass and metabolic activity, on the mortality in candidaemia. While the second aim was to examine the potential antifungal and/or adjuvant effect of farnesol against *C. auris*. To examine the effect of farnesol on *C. auris*, we performed experiments focusing on growth, biofilm production ability, triazole susceptibility and virulence.

Based on biofilm-related epidemiological experiments, intermediate/high biofilm mass was associated with significantly higher mortality (61%). The mortality was significantly higher in infections caused by *Candida* strains producing biofilms with intermediate/high metabolic activity (62% vs. 33%, $p<0.01$). In *C. auris*-related experiments, farnesol treatment showed a concentration dependent inhibition in terms of biofilm forming ability of *C. auris*; however, it did not inhibit significantly the biofilm development at 24 h. Moreover, 300 μM farnesol exerted a marked decrease in metabolic activity against one-day-old biofilms between 2 and 24 h ($p<0.001$). The interaction between azoles and farnesol showed synergism (FICI ranges from 0.038 to 0.375) against one-day-old biofilms. Daily 75 μM farnesol treatment decreased the fungal burden in an immunocompromised murine model of disseminated candidiasis ($p<0.01$).

In summary, our results provide evidence that *Candida* biofilms, especially with intermediate/high metabolic activity, are related to higher mortality in candidaemia. Furthermore, farnesol shows a promising therapeutic or adjuvant potential in traditional or alternative therapies against *C. auris*.

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

Registry number: DEENK/435/2021.PL
Subject: PhD Publication List

Candidate: Eszter Vitális
Doctoral School: Doctoral School of Pharmacy

List of publications related to the dissertation

1. **Vitális, E.**, Nagy, F., Tóth, Z., Forgács, L., Bozó, A., Kardos, G., Majoros, L., Kovács, R. L.: Candida biofilm production is associated with higher mortality in patients with candidaemia. *Mycoses. 63* (4), 352-360, 2020.
   DOI: http://dx.doi.org/10.1111/myc.13049
   IF: 4.377
2. Nagy, F., **Vitális, E.**, Jakab, Á., Borman, A. M., Forgács, L., Tóth, Z., Majoros, L., Kovács, R. L.: In vitro and in vivo effect of exogenous farnesol exposure against Candida auris. *Front. Microbiol. 11*, 1-12, 2020.
   DOI: http://dx.doi.org/10.3389/fmicb.2020.00957
   IF: 5.64

List of other publications

3. Nagy, G. G., Tudlik, Z., Gergely, L., Kónya, J., Orosi, P., Rákóczi, É., Szabó, J., Várvölgyi, C., **Vitális, E.**, Paragh, G.: A székletmikrobiota-transzplantáció technológiájának és minőségirányítási hátterének újragondolása a SARS-CoV-2 víruspandémia kapcsán. *Orv. hetil. 161* (44), 1858-1871, 2020.
   DOI: http://dx.doi.org/10.1556/650.2020.32023
   IF: 0.54
4. Velkey, B., **Vitális, E.**, Vitális, Z.: Spontán bakteriális peritonitis. *Orv. hetil. 158* (2), 50-57, 2017.
   DOI: http://dx.doi.org/10.1556/650.2017.30637
   IF: 0.322
5. **Vitális, E.**: Perioperatív antibiotikum-profilaxis. In: Perioperatív betegellátás. Szerk.: Tassonyi Edömér, Fülesdi Béla, Molnár Csilla, Medicina Könyvkiadó, Budapest, 350-356, 2016.

DEBRECENI EGYETEM Egyetemi és Nemzeti Könyvtár

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

6. Nemes, B. Á., Gelley, F., Dabasi, E., Gámán, G., Fehérvári, I., Görög, D., Kóbori, L., Fazakas, J., **Vitális, E.**, Doros, A., Gálffy, Z., Máthé, Z.: Bakteriális infekciók májátültetés után. *Orvosi Hetilap. 156* (34), 1366-1382, 2015.
DOI: http://dx.doi.org/10.1556/650.2015.30204
IF: 0.291

7. Fazekas, B., Mohácsi, A., Gyöngyösi, Z., **Vitális, E.**, Simon, É., Molnár, C., Fülesdi, B.: A maghőmérséklet transoesophagealis mérésének megbízhatósága félig zárt és zárt narkózis rendszerrel végzett anesztéziák során.
Anaesthesiol. Intenziv Ther. 38 (3), 121-126, 2008.

8. Molnár, C., Kovács, Z., Tankó, B., Fülep, Z., **Vitális, E.**, Sárkány, P., Fülesdi, B.: Különböző elven működő anesztézia mélység mérő monitorok klinikai alkalmazásának összehasonlítása idegsebészeti beavatkozások során = Comparison of two different indices used in anaesthesia depth monitors during neurosurgical operations.
Anaesthesiol. Intenziv Ther. 37 (3), 115-120, 2007.

Total IF of journals (all publications): 11,17
Total IF of journals (publications related to the dissertation): 10,017

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

09 September, 2021

DEBRECENI EGYETEM * Egyetemi és Nemzeti Könyvtár *