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Impact of *Candida albicans* hyphal wall protein 1 (*HWP1*) genotype on biofilm production and fungal susceptibility to microglial cells

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ABSTRACT

The hyphal wall protein 1 (*HWP1*) gene of *Candida albicans* encodes for a fungal cell wall protein, required for hyphal development and yeast adhesion to epithelial cells; yet, its role in pathogenesis remains largely unknown. In the present study, we analyzed two *C. albicans* laboratory strains, the DAY286 (*HWP1/HWP1*) and the null mutant FJS24 (*hwp1/hwp1*) and six clinical isolates [3 harbouring the homozygous *HWP1* gene (*HWP1/HWP1*) and 3 the heterologous gene (*HWP1/hwp1*)]. Biofilm production, fungal *HWP1* mRNA levels and ultrastructural morphology were investigated; also, the susceptibility of these strains to microglial cells was evaluated, in terms of fungal damage and immune cell-mediated secretory response. When comparing the two laboratory strains, biofilm was produced to a similar extent independently on the genetic background, while the susceptibility to microglial cell-mediated damage was higher in the *hwp1/hwp1* mutant than in the *HWP1/HWP1* counterpart. Also, transmission electron microscopy revealed differences between the two in terms of abundance in surface adhesin-like structures, fungal cell wall shape and intracellular granules. When comparing the clinical isolates grouped according to their *HWP1* genotype, reduced biofilm production and increased susceptibility to microglial cell-mediated damage occurred in the *HWP1/hwp1* isolates with respect to the *HWP1/HWP1* counterparts; furthermore, upon exposure to microglial cells, the *HWP1/HWP1* isolates, but not the *HWP1/hwp1* counterpart, showed enhanced *HWP1* mRNA levels. Finally, both laboratory and clinical isolates exhibited reduced ability to stimulate TNFα and nitric oxide production by microglial cells in the case of heterozygous or null mutant *HWP1* genotype.

Overall, these data indicate that *C. albicans HWP1* genotype influences pathogen morphological structure as well as its interaction with microglial cells, while fungal biofilm production results unaffected, thus arguing on its role as virulence factor that directly affects host mediated defences.

1. Introduction

Candida albicans, a commensal of human mucosa in healthy people, behaves as an opportunistic pathogen in critical patients, where it causes deep-seated life-threatening infections [1]. In particular, invasive candidiasis (IC) affects 9% of patients in the intensive care units and 2–20% of preterm newborns, with a mortality ranging from 20 to 50% [2,3]. Medical devices, such as cardiovascular and urinary catheters, dialysis access and ventriculoperitoneal shunts are considered to be a crucial point of entry for *Candida* in these clinical settings [4–6], where, though relatively rare, meningoencephalitis may also occur as a severe complication of deep-seated candidiasis [2,3,7]. To date, little is known on the events driving the onset of such deep-seated infections; increasing attention is being focused on fungal capacity to produce biofilm directly onto medical devices as a first and critical step towards host invasion [8,9].

In line with this, initial evidence indicates that biofilm-producing *C. albicans* isolates are less susceptible to host innate

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Glossary

HWP1	hyphal wall protein 1
HWP1/HWP1	strain harbouring the homozygous *HWP1* genotype
HWP1/hwp1	strain harbouring the heterozygous *HWP1* genotype
hwp1/hwp1	null mutant strain for *HWP1* gene
IMH3	inosine-5′-monophosphate dehydrogenase fragment
NO	nitrite
OD	optical density
PBS	phosphate-buffered saline
CV	crystal violet
XTT	2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide
YPD	yeast peptone dextrose medium

immune defences than biofilm-non producing counterparts [10,11]. Thus, biofilm formation is assumed to work as an immune escape mechanism that, in conjunction with reduced susceptibility to antifungal agents, may greatly support the persistence of implant-associated *C. albicans* infections [6]. Moreover, it has been recently shown that *Candida* biofilm can act *in vitro* as a reservoir of infectious viruses [12] further expanding the potential implications of fungal biofilms in clinical settings.

Numerous *C. albicans* gene products have been identified as relevant to biofilm development onto abiotic surfaces *in vitro* [13,14], while information on genes that enable biofilm formation *in vivo* has begun to emerge only recently [15,16]. A library of *C. albicans* mutants, defective in specific genetic traits, has been partially screened [13]; by this approach, several genes including *BCR1*, *ALS1*, *ALS3*, *ECE1* and *HWP1*, have been shown to be required for biofilm formation [16,17]. In particular, the gene *HWP1* is known to encode a major *C. albicans* protein involved in several functions, including cell wall assembly, intracellular signalling and hyphal development; moreover, *HWP1* promotes binding of *Candida* to epithelial cells, as the initial step of colonization [18–24]. Furthermore, some evidence exists on the pathogenic role of *HWP1 in vivo*; indeed, as assessed by a murine model of systemic candidiasis, it has been shown that the heterologous and the null mutant strains exhibit reduced and no virulence, respectively, when compared to the wild type control strain [19,25].

Not only *Candida*, with its plethora of finely regulated virulence traits, but also the first line host defences critically impact on the onset and outcome of candidiasis. Initial studies provided relevant information on the role of brain macrophages in the control of meningoencephalitis. In particular, by a murine model of intracerebral infection, it has been shown that microglial cells exert a protective role [26], through efficient phagocytosis and anti-*Candida* activity, whose biomolecular mechanisms have partially been defined [27–30]. Furthermore, microglial cell-mediated defences are affected by *C. albicans* peculiarities, such as the genotype profile (b versus c) and the dimorphic transition from yeast-to-hyphal form [31–33].

Here, by means of two *C. albicans* laboratory strains (the reference strain DAY286 and its isogenic *hwp1/hwp1* mutant, FJS24 strain) and six clinical isolates (3 harbouring the homozygous *HWP1/HWP1* genotype and 3 the heterozygous *HWP1/hwp1*), the role of *HWP1* genotype has been evaluated in terms of biofilm production and *Candida*-microglial cells interaction.

2. Materials and methods

2.1. C. albicans

We used two laboratory strains: the reference DAY286 (*HWP1/HWP1*) and its isogenic engineered counterpart, the null mutant FJS24 (*hwp1/hwp1)* [13,16]; such strains were purchased from the Fungal Genetics Stock Center (Kansas City, Missouri, USA) [34]. We also used six clinical isolates of *C. albicans*, recently collected during an Italian National Survey on invasive fungal infections [35]; such isolates had been characterized according to their *HWP1* gene polymorphism [36]. In particular, three isolates (lab names: Ca50vr, Ca23tv and Ca4no) harboured the homozygous genotype *HWP1/HWP1* and three isolates (laboratory names: Ca24ba, Ca48va and Ca17vr) were bearing the heterozygous genotype *HWP1/hwp1*. Long-term storage of the isolates was carried out in 20% glycerol at −80 °C. Before testing, a loopful of cells from stock cultures were inoculated in yeast peptone dextrose liquid medium (YPD, Oxoid, Hampshire, UK) and incubated overnight in an orbital shaker at 30 °C. Then, yeast cells were washed twice with sterile phosphate-buffered saline (PBS, EuroClone, Wetherby, UK), counted and suspended in medium at the desired concentration (10^5 or 10^6 cells/ml).

2.2. Cell lines

The previously established murine microglial cell line BV2 [31] and the K562 erythroid cell line were maintained by biweekly passages in RPMI 1640 (Gibco, Grand Island, NY, USA), supplemented with 10% heat inactivated foetal bovine serum (Defined Hyclone, Logan, Utah, USA), 50 mg/ml gentamicin (EuroClone) and 2 mM L-glutamine (EuroClone), hereafter referred to as complete medium. Experimental cultures were set at a concentration of 5×10^5 cells/ml in complete medium on the day before each experiment.

2.3. Biofilm formation

Biofilm formation was obtained on 96-well culture plates, according to a previously described protocol [37] with minor modifications. Briefly, *C. albicans* yeast cells (10^5 cells/ml) were incubated at 37 °C for 24 h, in complete medium and in presence of 5% CO_2. Then, biofilm production was evaluated by crystal violet (CV) assay or 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT, Sigma, St Louis, MO, USA) reduction assay, as detailed elsewhere [37]. For the CV assay, absorbance was measured spectrophotometrically by the SunRise Microplate Reader (Sunrise, Tecan, Salzburg, Austria) at 540 nm optical density (OD_{540}) and the colour intensity was proportional to biofilm biomass. For the XTT assay, the absorbance was measured spectrophotometrically at 450 nm with 620 nm as reference wavelength and the results, expressed as $OD_{450/620}$, were correlated with cellular metabolic activities within the biofilm. Each sample was assessed in 6-replicates.

2.4. Effects of microglial cells on C. albicans biofilm formation

In order to assess the effects of microglial cells on biofilm formation, complete medium and BV2 cells (10^5 cells/ml) were added to *C. albicans* yeast cells that had been pre-incubated at 37 °C in 5% CO_2 for 3 h. In parallel groups, K562 cells (10^5 cells/ml) were added as controls. Operationally, the 3 h pre-incubation was performed to allow fungal adhesion, as initial step in biofilm production. After additional 24 and 48 h incubation, microglial cells were lysed hypotonically, fungal biofilm formation (mass) and its metabolic

activity were assessed by CV and XTT assays, respectively, in the presence or absence of BV2 and of K562 cells. Results were expressed as OD values (CV and XTT assays). Moreover, based on the literature [10], the percentage of fungal damage was calculated employing the data obtained with the XTT assay, according to the formula: $100 \times [1 - (OD_{Candida + BV2}/OD_{Candida\ alone})]$.

2.5. Assessment of fungal gene expression

C. albicans yeast cells (10^6 cells/ml) were pre-incubated in 25 ml cell culture flasks in complete medium at 37 °C, in presence of 5% CO_2, for 3 h. Then, BV2 cells (10^6 cells/ml) or complete medium were added and the flasks were further incubated. After 1 h, microglial cells were lysed hypotonically and RNA was extracted using the TRIzol reagent (Invitrogen, CA, USA), according to manufacturer protocol. Then, purity and concentration of RNA extracts were measured by a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and up to 2 ng were retrotranscribed by the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA), in a final volume of 25 μl. Quantitative real-time PCR was performed in 48 well plates using the ABI StepOne apparatus (Applied Biosystems) and the Fast SYBR Green Master Mix (Applied Biosystems). Two primer sets were designed for the target gene *HWP1* and the housekeeping gene inosine-5′-monophosphate dehydrogenase fragment (*IMH3*) using the Primer Express 2.0 software (Applied Biosystems); primer sequences were as follows:

HWP1 (5′-TGCTATCGCTTATTACATGTTATC-3′, and 5′-GAGCTTCTTCTGTTTCACCTTGAC−3′);

IMH3 (5′-TATTCATATGGCATTATTGGGTGGTA-3′, and 5′-AACCATTTCTGCTTGTTCTTCAGA-3′).

The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The control gene *IMH3* was used to normalize the results.

2.6. Cytokine measurement and nitric oxide determination

Assays were performed as previously detailed [33]. Briefly, *C. albicans* yeast cells, 10^5 cells/ml in 24-well plates, were pre-incubated for 3 h in complete medium at 37 °C and in presence of 5% CO_2. Then, BV2 cells (10^6 cells/ml) or LPS (1 μg/ml, Sigma), the latter used as a positive control, were added to yeast cultures for additional 6 and 24 h. Supernatants were then collected and assayed for TNFα content, by ELISA (R&D Systems, Minneapolis, USA) according to manufacturer's recommendations. The reaction was read using the SunRise Microplate Reader ($OD_{450/540}$). Each sample was run in triplicate and results were expressed as pg/ml of cytokine. In parallel, the concentration of nitrite (NO) in the same supernatants was also measured using the Griess reagent, as previously described [33].

2.7. Transmission electron microscopy (TEM)

Samples for TEM analysis were processed as detailed elsewhere [38]. In particular, *C. albicans* yeast cells (10^6 cells/ml) were pre-incubated in 6 well plates in complete medium at 37 °C, in presence of 5% CO_2, for 3 h. Then, BV2 cells (10^6 cells/ml) or complete medium were added and incubation was carried out for further 24 h. Then, monolayers were washed with PBS, scraped and centrifuged at 10,000× *g*. The resulting pellets were fixed overnight with 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in Tyrode's buffer (Electron Microscopy Sciences), post-fixed for 2 h in 1% osmium tetroxide (Electron Microscopy Sciences), dehydrated and embedded in Spurr resin (Electron Microscopy Sciences). Semi-thin sections, obtained through the whole thickness of the pellets, were stained with toluidine blue and observed with a Zeiss Axiophot (Carl Zeiss, Jena, Germany) light microscope. Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a JEM 2010 (Jeol, Tokyo) transmission electron microscopy.

2.8. Statistical analysis

Statistical analysis was performed by the Student's *t*-test. Data in figures and table are expressed as mean or median values ± standard deviation (SD) or standard error (SE) of three to five independent experiments, unless otherwise specified.

3. Results

3.1. Biofilm production by the DAY286 (reference) and the FJS24 hwp1/hwp1 mutant strains

To assess the involvement of the *HWP1* genotype on biofilm production, two widely described laboratory strains [13,16], the DAY286 (reference) and its *hwp1/hwp1* mutant (FJS24), were investigated according to the protocols described in Materials and methods. Initially, the two strains were investigated for their ability to produce biofilm, according to previously established protocols [37]. As assessed by CV assay (Fig. 1), both strains achieved high and comparable levels of OD_{540} after 24 h in complete medium. Moreover, no significant changes/differences in OD_{540} levels were observed after 48 h. Similar patterns of results, confirming no differences between the two strains, were also observed in parallel groups, as assessed by the XTT assay (data not shown).

3.2. Impact of microglial cells on DAY286 (reference) and hwp1/hwp1 mutant strains

The impact of BV2 microglial cells on biofilm formation by the two laboratory strains was evaluated. Briefly, *C. albicans* yeast cells were pre-incubated for 3 h in complete medium; then, BV2 cells were added and the plates incubated for additional 24 or 48 h. At each time point, the XTT assay was performed and the results were then expressed as percent of fungal damage by microglial cells, according to the established protocols [10]. As detailed in Fig. 2a, significant differences occurred between the two strains in terms of susceptibility to BV2 microglial cells; in particular, the null mutant showed levels of microglial cell-mediated damage significantly

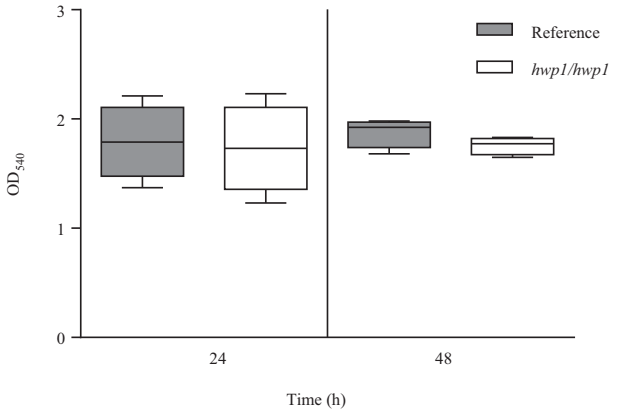

Fig. 1. Biofilm production by *C. albicans* strains DAY286 (reference) and *hwp1/hwp1* (mutant). Yeast cells (10^5 cells/ml), were pre-incubated for 3 h (in complete medium and in 5% CO_2), then BV2 cells were added (10^6 cells/ml). After 24 and 48 h of incubation, CV assay was performed. Columns represent the range of OD_{540} values. SD are also shown.

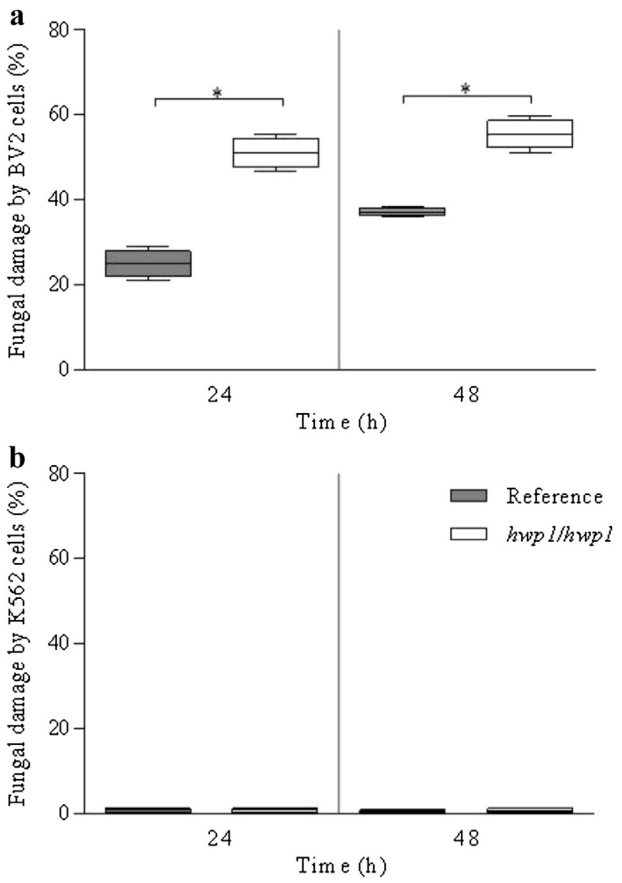

Fig. 2. Fungal damage induced by BV2 but no by K562 cells *on C. albicans* biofilm. Yeast cells (10^5 cells/ml), were pre-incubated for 3 h (in complete medium and in presence of 5% CO_2), then medium or cells (10^6 cells/ml) were added. After 24 and 48 h of incubation, XTT assay was performed. Box-plots represent the percentage of fungal damage, calculated according to the formula reported in Materials and methods section, when *Candida* cells were exposed to BV2 (a) or to K562 cells (b). SD are also shown. Asterisks denote statistically significant difference, (*) $p \leq 0.05$.

higher than the reference strain: 51% versus 24% at 24 h and 55% versus 37% at 48 h, respectively. When in parallel groups, the non-immuno-competent cell line K562 was included as negative control (Fig. 2b), no fungal damage was observed either at 24 or 48 h of incubation.

To further investigate the phenomenon, morphological analysis was performed. As detailed in Fig. 3, TEM images of *Candida* alone (incubated for 24 h in complete medium) revealed abundant and expanded adhesin-like structures on the surface of the DAY286 cells (Fig. 3a) but not on cells of the null mutant strain (Fig. 3b). Remarkable differences were also evident in terms of cell walls, which appeared loose and less compact in the null mutant strain with respect to the reference strain (see inserts in Fig. 3a and b). Furthermore, as assessed by optical microscopy (Fig. 4), after 24 h of co-culture, microglial cells appeared profoundly damaged, irrespective of the contact with either DAY286 (Fig. 4a) or *hwp1/hwp1* (Fig. 4b) *Candida* cells, while hyphal forms were more abundant in the DAY286 (Fig. 4a). In addition, large-size intracellular granules were especially evident in the reference strain (Fig. 4a vs b).

3.3. Biofilm production by HWP1/HWP1 and HWP1/hwp1 clinical isolates and their susceptibility to microglial cells

The *C. albicans* clinical isolates, three harbouring the *HWP1/HWP1* and three the *HWP1/hwp1* genotypes, were compared for their capacity to form biofilm when grown in complete medium. As assessed by the XTT assay (Fig. 5a), the *HWP1/HWP1* isolates displayed OD_{540} levels significantly higher than the *HWP1/hwp1* counterparts ($p < 0.05$); the phenomenon was evident and consistent both at 24 and 48 h; a similar trend of results was observed also by the CV assay (data not shown). Furthermore, the same *C. albicans* isolates were investigated for their susceptibility to microglial cells. Thus, *C. albicans* yeast cells were pre-incubated for 3 h in complete medium; then, microglial cells were added and the plates incubated for additional 24 or 48 h. At each time point, the XTT assay was performed and the results were then expressed as percent of fungal damage by microglial cells. As detailed in Fig. 5b, fungal damage was significantly higher in the *HWP1/hwp1* isolates than in the *HWP1/HWP1* counterparts at both time points (54% vs 26% at 24 h and 45% vs 20% at 48 h, respectively).

To better understand the interaction of the clinical isolates with the BV2 cells, these isolates were investigated by RT-PCR for the relative levels of *HWP1* gene expression in the presence and in the absence of microglial cells. Briefly, *C. albicans* yeast cells (10^6 cells/ml) were pre-incubated for 3 h in complete medium. Then, complete medium or BV2 cells (10^6 cells/ml) were added and the cultures were further incubated for 1 h. The relative levels of *HWP1* gene expression are showed in Fig. 5c. A consistent up-regulation of *HWP1* mRNA levels occurred in the group of *HWP1/HWP1* isolates incubated in the presence of microglial cells compared to the control group (*Candida* alone). By contrast, *HWP1/hwp1* isolates showed a limited though consistent down-regulation in the presence of microglial cells.

3.4. Microglial cell secretory response to C. albicans laboratory strains and clinical isolates

Finally, the profile of microglial cell secretory response to both laboratory strains and clinical isolates was investigated, according to previously established protocols [10,33]. Briefly, yeast cells were pre-incubated in complete medium for 3 h; then, BV2 cells were added for additional 6 and 24 h. In parallel groups, BV2 cells were exposed to LPS, used as positive control. Supernatants were harvested and assessed for TNFα and NO content. Table 1 shows TNFα and NO production by BV2 cells exposed to the two laboratory strains, the DAY286 and the mutant FJS24, and two clinical isolates, Ca50vr and Ca24ba, representative of the *HWP1/HWP1* and *HWP1/hwp1* genotypes, respectively. As expected, LPS greatly enhanced TNFα production by BV2 cells, with maximal levels being reached at 6 h. A time-dependent production of TNFα was also observed in BV2 cells exposed to *Candida* isolates. In particular, both DAY286 and Ca50vr (*HWP1/HWP1*) induced levels of TNFα significantly higher than their counterparts, the mutant FJS24 and Ca24ba (*HWP1/hwp1*). Concerning NO production, a significant increase occurred, at 24 h, only in BV2 cells exposed to DAY286 and Ca50vr (*HWP1/HWP1*).

4. Discussion

Here, by means of laboratory isogenic strains and clinical isolates, we show that *C. albicans HWP1* genotype does not affect biofilm formation *in vitro*, but rather it influences fungal susceptibility to microglial cells.

Increasing literature is being provided on fungal genetic traits that are possibly involved in biofilm formation and virulence [13,15–17]; construction of laboratory mutant strains as well as genotyping of clinical isolates has allowed direct associations between certain phenotypic peculiarities/functions and specific genotypic elements [19,20]. In particular, the *HWP1* gene appears to be involved in several pathogenic steps, including adherence to

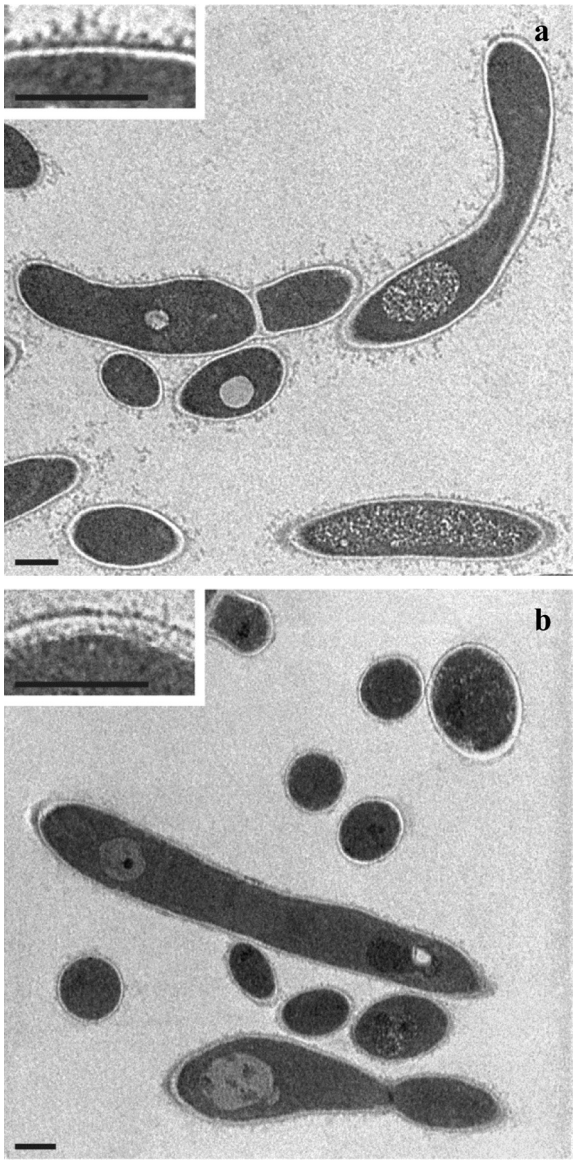

Fig. 3. Ultrastructural morphology of *C. albicans* strains DAY286 (reference) and *hwp1/hwp1* (mutant). Yeast cells (10^6 cells/ml), incubated for 24 h (in complete medium and in presence of 5% CO_2), were fixed and embedded in resin to allow observation of ultrathin sections by TEM. Representative image of (a) DAY286 reference strain and (b) *hwp1/hwp1* mutant strain. Bar = 1 μm.

epithelial cells and virulence in mice [19–22,24,25]. Recently, by a National Survey Study on invasive fungal infections, we obtained and partially characterized a collection of 142 clinical *C. albicans* isolates, whose profiles of *HWP1* genotype, biofilm production and antifungal drug susceptibility have been investigated [35]. Also, initial information has been provided on the relationship between ability to produce biofilm *in vitro* and virulence in *Galleria mellonella* [11]. With the aim of more in depth investigating the role of *HWP1* genotype on biofilm formation, the present study has been conducted employing i) two laboratory strains, the reference *C. albicans* DAY286 strain and its *HWP1* null mutant FJS24, and ii) six clinical isolates, harbouring the *HWP1/HWP1* and the *HWP1/hwp1* genotype. Preliminarily, *Candida* strains/isolates have been compared for their growth rate under different conditions, namely at different yeast cell concentrations and in the presence of complete medium or YPD, over a 48-h incubation period. As detailed in Supplementary Table S1, although the growth rates appear different in complete medium or YPD, yet, no statistically significant differences have been observed when comparing the DAY286 with its mutant FJS24 or the Ca50vr *HWP1/HWP1*with the Ca24ba *HWP1/hwp1* clinical isolates. As detailed in Fig. 1, the two laboratory strains produce biofilm to a similar extent, while differences are evident between *HWP1/HWP1* and *HWP1/hwp1* clinical isolates, with the latter being lower biofilm producers (Fig. 5a). In line with these data, Tsuchimori and coworkers [25] have recently shown that also other sets of engineered strains, differing in *HWP1* genotype, exhibit similar propensities to produce biofilm. In contrast, our results on clinical isolates reveal differences in biofilm formation between the two groups, operationally clustered in this study according to their *HWP1* genotype. The unexpected discrepancy observed with the clinical isolates raises the possibility that additional unexplored genotypic differences between homo- and heterozygous *HWP1* isolates may be responsible for the different behaviour, at least in terms of biofilm production. Possibly, a complete genotypic characterization of the clinical isolates may

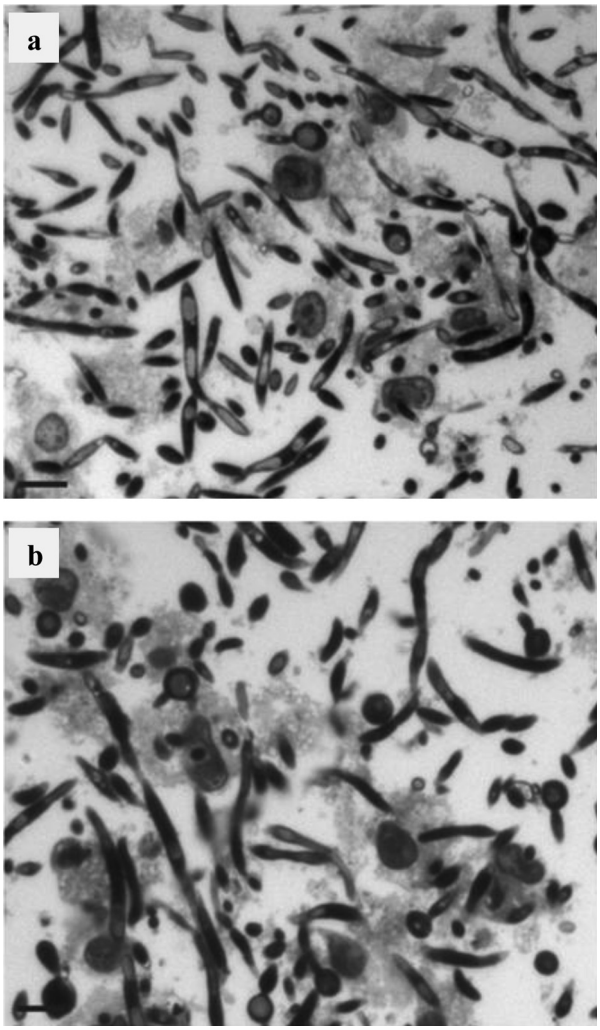

Fig. 4. Morphology of 24 h-old *Candida*-microglial cells co-cultures. *C. albicans* DAY286 (reference) and FJS24 (*hwp1/hwp1* mutant), at the 10^6 cells/ml cell density, were pre-incubated for 3 h (in complete medium and in 5% CO_2), then BV2 cells were added (10^6 cells/ml). After 24 h of incubation, samples were fixed and embedded in resin. Semi-thin sections were examined by light microscopy. Representative images of (a) DAY286 reference strain and (b) *hwp1/hwp1* FJS24 mutant strains, both exposed to BV2 cells. Bar = 10 μm.

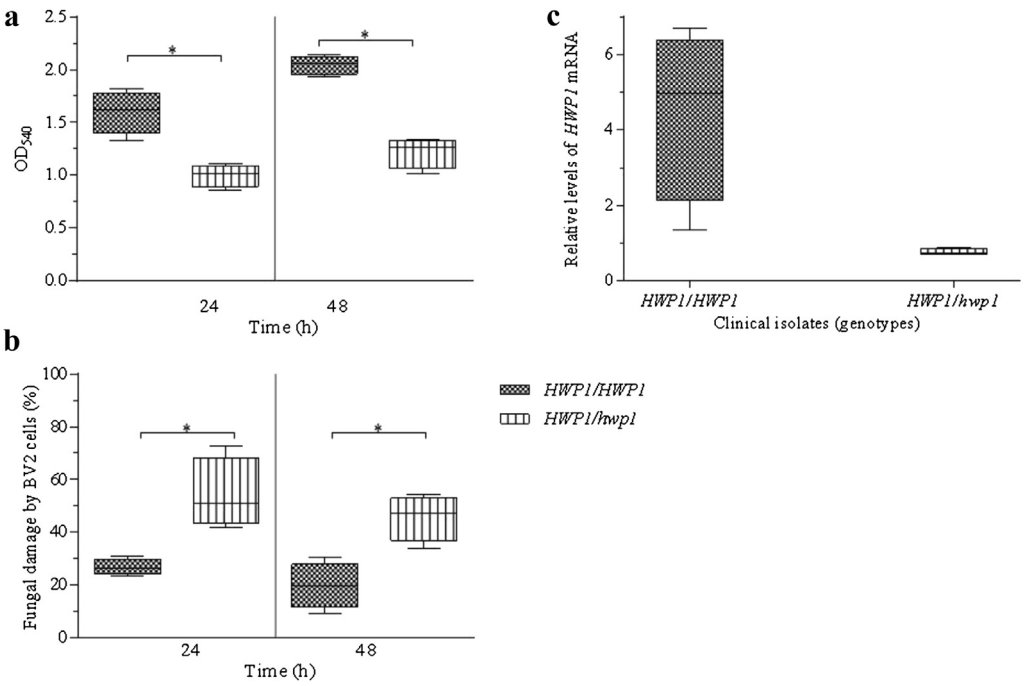

Fig. 5. Biofilm production by *C. albicans* clinical isolates and their susceptibility to microglial cells. Three *HWP1/HWP1* and 3 *HWP1/hwp1* isolates were assessed. Biofilm production (a) by yeast cells (10^5 cells/ml) incubated for 24 and 48 h, in complete medium, was evaluated by CV assay. Box-plots represent the range of OD_{540} values. For the evaluation of fungal susceptibility to BV2 cell, yeast cells (10^5 cells/ml) were pre-incubated for 3 h (in complete medium), then complete medium or BV2 cells were added (10^6 cells/ml) for 1, 24 or 48 h. After 24 and 48 h of incubation, XTT assay was performed and box-plots represent the percentage of fungal damage (b), calculated according to the formula detailed in Materials and methods section. In parallel, after 1 h of incubation, yeast RNA was extracted and *HWP1* gene expression was evaluated by RT-PCR (c). Box-plots represent the relative levels of *HWP1* mRNA expression. Mean values and SD are also shown. Asterisks denote statistically significant differences, (*) $p \leq 0.05$.

provide clear-cut information on this matter. At the moment, based on previous [25] and present findings, we favour the conclusion that *HWP1* genotype does not directly affect *Candida* ability to produce biofilm *in vitro*.

Previous studies demonstrate that microglial cells efficiently contrast *C. albicans* by phagocytosis and killing of the yeast cells [26,28,33]. Here, we show that such immune cells are also capable of affecting fungal biofilm and that the extent of such phenomenon is related to *Candida HWP1* genotype; interestingly, the impact of microglial cells on biofilm production is similar, both in clinical isolates and laboratory strains. In particular, when exposed to microglial cells for 24 h, both the engineered *hwp1/hwp1* mutant strain (Fig. 2a) and the heterozygous clinical *HWP1/hwp1* isolates (Fig. 5b) are damaged to a higher extent than their *HWP1/HWP1* counterparts; such differential profiles of susceptibility are retained also at later times (48 h), when the null mutant (Fig. 2a) and the heterozygous (Fig. 5b) are still significantly affected, while the homozygous, *HWP1/HWP1*, are no longer susceptible to microglial cell-mediated damage. Interestingly, non immuno-competent cells, such as the erythroid cell line K562, fail to exert any deleterious effect on fungal biofilm production (Fig. 2b), thus further strengthening the direct role of BV2 cells while other trivial events such as nutrient limitation or steric cell-to-cell interference may be ruled out. Overall, by assessing different parameters, namely fungal biomass and metabolic activity, the CV and XTT assays unanimously indicate that *Candida* biofilm production is significantly impaired by microglial cells. Our present findings are in line with a previous study demonstrating that phagocytes can induce biofilm damage

Table 1
TNFα and NO production by BV2 microglial cells exposed to laboratory strains and clinical isolates for 6 and 24 h.

BV2 cell exposure to:	TNFα (pg/ml)			NO (μM)		
	6 h	24 h		6 h	24 h	
Medium	23,40	23,40		3,00	3,39	
LPS (1 μg/ml)	5360,00	4685,10		3,39	8,93	
DAY286	299,01	340,56	*	4,62	13,20	*
FJS24 (*hwp1/hwp1*)	220,12	223,22	*	4,60	4,60	*
Ca50vr (*HWP1/HWP1*)	297,49	334,31	*	4,62	13,53	*
Ca24ba (*HWP1/hwp1*)	223,85	231,21	*	4,62	4,62	*

Candida yeast cells (10^5 cell/ml) were pre-incubated in complete medium for 3 h; then complete medium or BV2 cells were added and the plates were incubated for additional 6 or 24 h. TNFα and NO were subsequently measured in the cell-free supernatants, as detailed above. SD values were always less than 5% and therefore they have been omitted. * $p \leq 0.001$.

[10]. By contrast, it has been shown that human peripheral blood mononuclear cells may enhance the ability of *C. albicans* to form biofilm [39]. Differences in the experimental protocols employed (timing, cultural conditions, etc.) and in the immune cell types investigated may account for the apparent disagreement among studies aimed at defining the role of phagocytes on fungal biofilm production.

Here, as assessed by the clinical isolates, only the homozygous genotypes show a significant enhancement of *HWP1* mRNA levels, following *Candida* exposure to the microglial cells (Fig. 5c). Besides indicating a different *Candida* behaviour based on its *HWP1* genotype, these data also imply the capacity of *Candida* to re-program its own molecular machinery in response to the insult provided by the immune cells, at least in terms of *HWP1* transcript levels, thus further supporting the idea that *HWP1* should be considered as a virulence trait. In line with this conclusion, Staab and coworkers [18] provide initial evidence on the relevance of *HWP1* genotype on *Candida* adhesion to epithelial cells *in vitro*.

Concerning the secretory response of microglial cells to *C. albicans*, enhanced TNFα and NO production occurs when employing the homozygous *HWP1/HWP1* genotypes, both of laboratory and clinical origin, while little or no response is observed against the heterozygous or null mutant counterpart (Table 1). In line with our previous work [32], we show that *C. albicans* promotes an enhanced secretory response by microglial cells.

Whether structural or rather pathogenetic changes are involved, in such a microglial cell-related response, remains to be investigated. In any case, the present data provide the first evidence that microglial cells secretory response is differently modulated, depending upon *Candida HWP1* genotype.

Finally, morphological analysis reveals several differences between the *HWP1/HWP1* and *hwp1/hwp1 Candida* cells. In particular, the former express abundant adhesin-like structures, barely detectable in the null mutant, within which both cell wall consistency and thickness appear altered (Fig. 3). While expanding previous morphological data [24,40], these findings emphasize the dual role of *HWP1* protein, both as a surface adhesion molecule and as a protein important in maintaining cell wall integrity. Moreover, here we show that upon *Candida*-microglial cell co-culture, the *hwp1/hwp1* fungal bodies appear not only less numerous but also they show reduced intracellular granules (Fig. 4); to our opinion, such features can be reasonably explained as markers of microglial cell-induced stress/damage. Thus, taken together, the morphological peculiarities and the quantitative data obtained by XTT and CV assays underline the enhanced susceptibility of the null mutant to microglial cells.

Initial efforts are being made in dissecting the pathogenic role of *HWP1* in candidiasis. By a murine model of oro-esophageal candidiasis, *HWP1* genotype has been associated with a peculiar pathogen-mucosal cell interplay [19,41], while *HWP1* ablation has resulted in reduced virulence, as assessed by a systemic candidiasis model [25,42]. Here, we provide *in vitro* evidence that *HWP1* genotype does not directly influence *Candida* ability to produce biofilm, but it significantly affects microglial cell-mediated efficacy. Thus, we may envisage *in vivo* situations where *Candida HWP1* genotype plays a pathogenetic role as soon as the first line immune defences are called up as critical defence players.

Author contributions

Conceived and designed the experiments: CFO RGN EB. Performed the experiments: CFO EB BC DQ. Analyzed the data: CFO EB AA. Contributed reagents/materials/analysis tools: GM EB. Wrote the paper: CFO EB AA.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.micpath.2014.03.003.

References

[1] Pfaller MA, Diekema DJ. Epidemiology of invasive candidiasis: a persistent public health problem. Clin Microbiol Rev 2007;20:133–63. http://dx.doi.org/10.1038/nport.2008.141.
[2] Gullo A. Invasive fungal infections: the challenge continues. Drugs 2009;69:65–73. http://dx.doi.org/10.2165/11315530-000000000-00000.
[3] Cahan H, Deville JG. Outcomes of neonatal candidiasis: the impact of delayed initiation of antifungal therapy. Int J Pediatr 2011;2011:813871. http://dx.doi.org/10.1155/2011/813871.
[4] Ramage G, Martínez JP, López-Ribot JL. *Candida* biofilms on implanted biomaterials: a clinically significant problem. FEMS Yeast Res 2006;6:979–86. http://dx.doi.org/10.1111/j.1567-1364.2006.00117.x.
[5] Wenzel RP, Edmond MB. The evolving technology of venous access. N Engl J Med 1999;340:48–50. http://dx.doi.org/10.1056/NEJM199901073400109.
[6] Kojic EM, Darouiche RO. *Candida* infections of medical devices. Clin Microbiol Rev 2004;17:255–67.
[7] Sánchez-Portocarrero J, Martín-Rabadán P, Saldaña CJ, Pérez-Cecilia E. *Candida* cerebrospinal fluid shunt infection. Report of two new cases and review of the literature. Diagn Microbiol Infect Dis 1994;20:33–40.
[8] Ramage G, Saville SP, Thomas DP, López-Ribot JL. *Candida* biofilms: an update. Eukaryot Cell 2005;4:633–8. http://dx.doi.org/10.1128/EC.4.4.633-638.2005.
[9] Kumamoto CA, Vinces MD. Alternative *Candida albicans* lifestyles: growth on surfaces. Annu Rev Microbiol 2005;59:113–33. http://dx.doi.org/10.1146/annurev.micro.59.030804.121034.
[10] Katragkou A, Kruhlak MJ, Simitsopoulou M, Chatzimoschou A, Taparkou A, Cotten CJ, et al. Interactions between human phagocytes and *Candida albicans* biofilms alone and in combination with antifungal agents. J Infect Dis 2010;201:1941–9. http://dx.doi.org/10.1086/652783.
[11] Cirasola D, Sciota R, Vizzini L, Ricucci V, Morace G, Borghi E. Experimental biofilm-related *Candida* infections. Future Microbiol 2013;8:799–805. http://dx.doi.org/10.2217/fmb.13.36.
[12] Mazaheritehrani E, Sala A, Orsi CF, Neglia RG, Morace G, Blasi E, et al. Human pathogenic viruses are retained in and released by *Candida albicans* biofilm in vitro. Virus Res 2014;179:153–60. http://dx.doi.org/10.1016/j.virusres.2013.10.018.
[13] Nobile CJ, Mitchell AP. Regulation of cell-surface genes and biofilm formation by the *C. albicans* transcription factor Bcr1p. Curr Biol CB 2005;15:1150–5. http://dx.doi.org/10.1016/j.cub.2005.05.047.
[14] Thomas DP, Bachmann SP, Lopez-Ribot JL. Proteomics for the analysis of the *Candida albicans* biofilm lifestyle. Proteomics 2006;6:5795–804. http://dx.doi.org/10.1002/pmic.200600332.
[15] Harriott MM, Lilly EA, Rodriguez TE, Fidel Jr PL, Noverr MC. *Candida albicans* forms biofilms on the vaginal mucosa. Microbiol Read Engl 2010;156:3635–44. http://dx.doi.org/10.1099/mic.0.039354-0.
[16] Nobile CJ, Andes DR, Nett JE, Smith FJ, Yue F, Phan Q-T, et al. Critical role of Bcr1-dependent adhesins in *C. albicans* biofilm formation *in vitro* and *in vivo*. PLoS Pathog 2006;2:e63. http://dx.doi.org/10.1371/journal.ppat.0020063.
[17] Nobile CJ, Nett JE, Andes DR, Mitchell AP. Function of *Candida albicans* adhesin *Hwp1* in biofilm formation. Eukaryot Cell 2006;5:1604–10. http://dx.doi.org/10.1128/EC.00194-06.
[18] Staab JF, Bradway SD, Fidel PL, Sundstrom P. Adhesive and mammalian transglutaminase substrate properties of *Candida albicans Hwp1*. Science 1999;283:1535–8.
[19] Sundstrom P, Balish E, Allen CM. Essential role of the *Candida albicans* transglutaminase substrate, hyphal wall protein 1, in lethal oroesophageal candidiasis in immunodeficient mice. J Infect Dis 2002;185:521–30. http://dx.doi.org/10.1086/338836.
[20] Naglik JR, Fostira F, Ruprai J, Staab JF, Challacombe SJ, Sundstrom P. *Candida albicans HWP1* gene expression and host antibody responses in colonization and disease. J Med Microbiol 2006;55:1323–7. http://dx.doi.org/10.1099/jmm.0.46737-0.
[21] Biswas S, Van Dijck P, Datta A. Environmental sensing and signal transduction pathways regulating morphopathogenic determinants of *Candida albicans*. Microbiol Mol Biol Rev 2007;71:348–76. http://dx.doi.org/10.1128/MMBR.00009-06.
[22] Chaffin WL. *Candida albicans* cell wall proteins. Microbiol Mol Biol Rev MMBR 2008;72:495–544. http://dx.doi.org/10.1128/MMBR.00032-07.
[23] Finkel JS, Mitchell AP. Genetic control of *Candida albicans* biofilm development. Nat Rev Microbiol Feb 2011;9(2):109–18. http://dx.doi.org/10.1038/nrmicro2475.

[24] de Groot PWJ, Bader O, de Boer AD, Weig M, Chauhan N. Adhesins in human fungal pathogens: glue with plenty of stick. Eukaryot Cell 2013;12:470–81. http://dx.doi.org/10.1128/EC.00364-12.
[25] Tsuchimori N, Sharkey LL, Fonzi WA, French SW, Edwards Jr JE, Filler SG. Reduced virulence of *HWP1*-deficient mutants of *Candida albicans* and their interactions with host cells. Infect Immun 2000;68:1997–2002.
[26] Blasi E, Mazzolla R, Pitzurra L, Barluzzi R, Bistoni F. Protective effect of picolinic acid on mice intracerebrally infected with lethal doses of *Candida albicans*. Antimicrob Agents Chemother 1993;37:2422–6.
[27] Blasi E, Pitzurra L, Puliti M, Mazzolla R, Barluzzi R, Saleppico S, et al. Different events involved in the induction of macrophage tumor necrosis factor by *Candida albicans* and lipopolysaccharide. Cell Immunol 1994;157:501–9. http://dx.doi.org/10.1006/cimm.1994.1245.
[28] Blasi E, Mazzolla R, Barluzzi R, Bistoni F. Microglial cell-mediated anti-*Candida* activity: temperature, ions, protein kinase C as crucial elements. J Neuroimmunol 1991;34:53–60.
[29] Blasi E, Mucci A, Neglia R, Pezzini F, Colombari B, Radzioch D, et al. Biological importance of the two Toll-like receptors, TLR2 and TLR4, in macrophage response to infection with *Candida albicans*. FEMS Immunol Med Microbiol 2005;44:69–79.
[30] Puliti M, Radzioch D, Mazzolla R, Barluzzi R, Bistoni F, Blasi E. Influence of the Bcg locus on macrophage response to the dimorphic fungus *Candida albicans*. Infect Immun 1995;63:4170–3.
[31] Blasi E, Barluzzi R, Bocchini V, Mazzolla R, Bistoni F. Immortalization of murine microglial cells by a v-raf/v-myc carrying retrovirus. J Neuroimmunol 1990;27:229–37.
[32] Blasi E, Pitzurra L, Puliti M, Lanfrancone L, Bistoni F. Early differential molecular response of a macrophage cell line to yeast and hyphal forms of *Candida albicans*. Infect Immun 1992;60:832–7.
[33] Neglia R, Colombari B, Peppoloni S, Orsi C, Tavanti A, Senesi S, et al. Adaptive response of microglial cells to *in vitro* infection by *Candida albicans* isolates with different genomic backgrounds. Microb Pathog 2006;41:251–6. http://dx.doi.org/10.1016/j.micpath.2006.08.001.
[34] McCluskey K. The fungal genetics stock Center: from molds to molecules. Adv Appl Microbiol 2003;52:245–62.
[35] Morace G, Borghi E, Iatta R, Amato G, Andreoni S, Brigante G, et al. Antifungal susceptibility of invasive yeast isolates in Italy: the GISIA3 study in critically ill patients. BMC Infect Dis 2011;11:130. http://dx.doi.org/10.1186/1471-2334-11-130.
[36] Padovan ACB, Chaves GM, Colombo AL, Briones MRS. A novel allele of *HWP1*, isolated from a clinical strain of *Candida albicans* with defective hyphal growth and biofilm formation, has deletions of Gln/Pro and Ser/Thr repeats involved in cellular adhesion. Med Mycol Off Publ Int Soc Hum Anim Mycol 2009;47:824–35. http://dx.doi.org/10.3109/13693780802669574.
[37] Pierce CG, Uppuluri P, Tristan AR, Wormley Jr FL, Mowat E, Ramage G, et al. A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing. Nat Protoc 2008;3:1494–500. http://dx.doi.org/10.1038/nport.2008.141.
[38] Quaglino D, Ha HR, Duner E, Bruttomesso D, Bigler L, Follath F, et al. Effects of metabolites and analogs of amiodarone on alveolar macrophages: structure-activity relationship. Am J Physiol Lung Cell Mol Physiol 2004;287:L438–47. http://dx.doi.org/10.1152/ajplung.00434.2003.
[39] Chandra J, McCormick TS, Imamura Y, Mukherjee PK, Ghannoum MA. Interaction of *Candida albicans* with adherent human peripheral blood mononuclear cells increases *C. albicans* biofilm formation and results in differential expression of pro- and anti-inflammatory cytokines. Infect Immun 2007;75:2612–20. http://dx.doi.org/10.1128/IAI.01841-06.
[40] Plaine A, Walker L, Da Costa G, Mora-Montes HM, McKinnon A, Gow NAR, et al. Functional analysis of *Candida albicans* GPI-anchored proteins: roles in cell wall integrity and caspofungin sensitivity. Fungal Genet Biol 2008;45:1404–14. http://dx.doi.org/10.1016/j.fgb.2008.08.003.
[41] Balish E, Warner T, Pierson CJ, Bock DM, Wagner RD. Oroesophageal candidiasis is lethal for transgenic mice with combined natural killer and T-cell defects. Med Mycol Off Publ Int Soc Hum Anim Mycol 2001;39:261–8.
[42] Sundstrom P, Cutler JE, Staab JF. Reevaluation of the role of HWP1 in systemic candidiasis by use of *Candida albicans* strains with selectable marker URA3 targeted to the ENO1 locus. Infect Immun 2002;70:3281–3. http://dx.doi.org/10.1086/338836.