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 secretary 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, meningocerephalitis 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
immune defences than biofilm-non producing counterparts [10,11]. Thus, biofilm formation is assumed to work as an 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 meningocencephalitis. 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, FSJ24 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 FSJ24 (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% CO2. Then, biofilm production was evaluated by crystal violet (CV) assay or 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-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_{540/620}, were corrected 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% CO2 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

### 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-sulphophenyl)-2H-tetrazolium-5-carboxanilide
- **YPD**: yeast peptone dextrose medium
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: $100 \times \left[1 - \frac{\text{OD}_{\text{Candida}} + \text{BV2}}{\text{OD}_{\text{Candida alone}}}\right]$.

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). Two primer sets were designed for the target gene *HWP1* and the housekeeping gene *β-actin* 5′-monophosphate dehydrogenase fragment (*IMH3*) using the Primer Express 2.0 software (Applied Biosystems); primer sequences were as follows:

*HWP1* (5′-TGCATGCCTATATGATATATATC-3′, and 5′-GAGCTTCCTGCCTACCCGCACGAC-3′);

*IMH3* (5′-TATTCATGCGATTGCGGTGTA-3′, and 5′-AACCATTTGCCTGCCTACAGA-3′).

The relative expression levels were calculated using the 2$^{-ΔΔCt}$ 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 ($\text{OD}_{490/540}$). 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 Axioshot (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...
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 OD540 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 (10⁶ cells/ml) were pre-incubated for 3 h in complete medium. Then, complete medium or BV2 cells (10⁶ 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 upregulation 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
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 \textit{C. albicans} isolates, whose profiles of \textit{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 \textit{Galleria mellonella} [11]. With the aim of more in depth investigating the role of \textit{HWP1} genotype on biofilm formation, the present study has been conducted employing i) two laboratory strains, the reference \textit{C. albicans} DAY286 strain and its \textit{HWP1} null mutant FJS24, and ii) six clinical isolates, harbouring the \textit{HWP1}/\textit{HWP1} and the \textit{HWP1}/\textit{hwp1} genotype. Preliminary, \textit{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 \textit{HWP1}/\textit{HWP1} with the Ca24ba \textit{HWP1}/\textit{hwp1} clinical isolates. As detailed in Fig. 1, the two laboratory strains produce biofilm to a similar extent, while differences are evident between \textit{HWP1}/\textit{HWP1} and \textit{HWP1}/\textit{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 \textit{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 \textit{HWP1} genotype. The unexpected discrepancy observed with the clinical isolates raises the possibility that additional unexplored genotypic differences between homo- and heterozygous \textit{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. 3. Ultrastructural morphology of \textit{C. albicans} strains DAY286 (reference) and \textit{hwp1}/\textit{hwp1} (mutant). Yeast cells (10^6 cells/ml), incubated for 24 h (in complete medium and in presence of 5% CO2), were fixed and embedded in resin to allow observation of ultrathin sections by TEM. Representative image of (a) DAY286 reference strain and (b) \textit{hwp1}/\textit{hwp1} mutant strain. Bar = 1 μm.](image)

![Fig. 4. Morphology of 24 h-old \textit{Candida}-microglial cells co-cultures. \textit{C. albicans} DAY286 (reference) and FJS24 (\textit{hwp1}/\textit{hwp1} mutant), at the 10^6 cells/ml cell density, were pre-incubated for 3 h (in complete medium and in 5% CO2), 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) \textit{hwp1}/\textit{hwp1} FJS24 mutant strains, both exposed to BV2 cells. Bar = 10 μm.](image)
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.

![Figure 5](image)

**Table 1**

<table>
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<th>BV2 cell exposure to</th>
<th>TNFα (pg/ml) 6 h</th>
<th>TNFα (pg/ml) 24 h</th>
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<th>NO (μM) 24 h</th>
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<td>231,21</td>
<td>4,62</td>
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Candida yeast cells (10^6 cells/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 ≤ 0.001.
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. 4c). 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 [12], 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.

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