

# Faster *Cryptococcus* melanization increases virulence in experimental and human cryptoccosis

1 Herdson Renney de Sousa<sup>1</sup>, Getúlio Pereira de Oliveira Júnior<sup>2</sup>, Stefânia de Oliveira Frazão<sup>3</sup>,  
2 Kaio Cesar de Melo Gorgonha<sup>1</sup>, Camila Pereira Rosa<sup>1</sup>, Emânuella Melgaço Garcez<sup>1</sup>, Joaquim  
3 Lucas Junior<sup>4</sup>, Amabel Fernandes Correia<sup>5</sup>, Waleriano Ferreira de Freitas<sup>1</sup>, Higor Matos  
4 Borges<sup>1</sup>, Hugo Costa Paes<sup>1</sup>, Luciana Trilles<sup>6</sup>, Marcia dos Santos Lazera<sup>6</sup>, Vitor Laerte Pinto  
5 Junior<sup>4</sup>, Maria Sueli Soares Felipe<sup>7</sup>, Arturo Casadevall<sup>8</sup>, Ildinete Silva-Pereira<sup>3</sup>, Patrícia  
6 Albuquerque<sup>3,9</sup>, André Moraes Nicola<sup>1,7,\*</sup>.

7 <sup>1</sup>Faculty of Medicine, University of Brasília, Brasília, DF, Brazil.

8 <sup>2</sup>Division of Allergy and Inflammation, Department of Medicine, Beth Israel Deaconess Medical  
9 Center, Harvard Medical School, Boston, MA, United States.

10 <sup>3</sup>Laboratory of Molecular Biology of Pathogenic Fungi, Institute of Biological Sciences, University  
11 of Brasília, Brasília, DF, Brazil.

12 <sup>4</sup>Oswaldo Cruz Foundation (Fiocruz – Brasília), Brasília, DF, Brazil.

13 <sup>5</sup>Central Public Health Laboratory (Lacen-DF), Brasília, DF, Brazil.

14 <sup>6</sup>Mycology Laboratory, Evandro Chagas National Institute of Infectious Diseases, Oswaldo Cruz  
15 Foundation (Fiocruz – Rio de Janeiro), Rio de Janeiro, RJ, Brazil

16 <sup>7</sup>Graduate Program in Genomic Sciences and Biotechnology, Catholic University of Brasília,  
17 Brasília, DF, Brazil.

18 <sup>8</sup>Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of  
19 Public Health, Baltimore, MD, USA.

20 <sup>9</sup>Faculty of Ceilândia, University of Brasília, Brasília, DF, Brazil.

21  
22 **Corresponding author:**

23 Name: André Moraes Nicola

24 Address: Campus Universitário Darcy Ribeiro, Núcleo de Medicina Tropical, sala 40. Brasília-DF,  
25 Brazil. 70910-900.

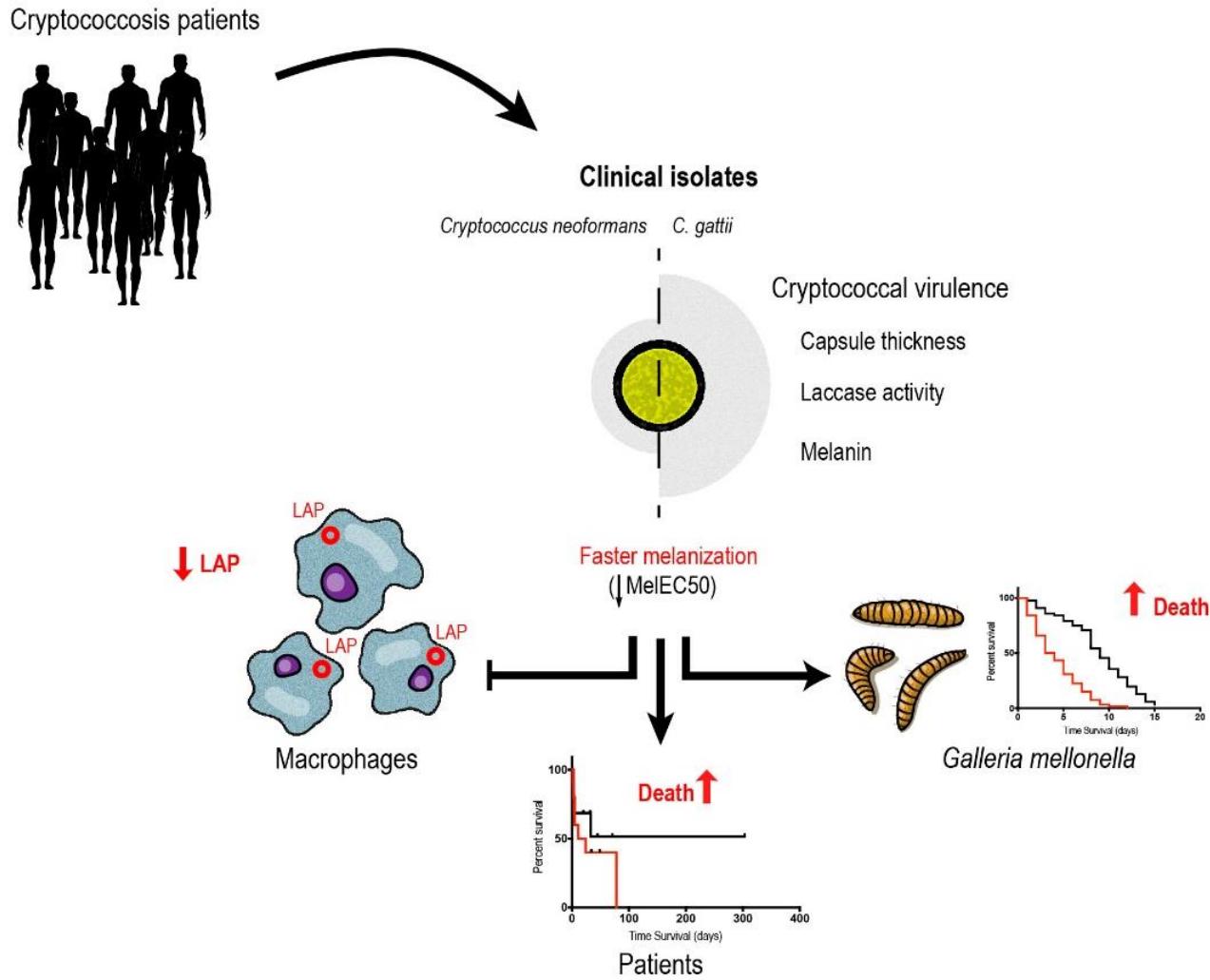
26 Phone: +55 61 992619048

27 E-mail: [amnicola@unb.br](mailto:amnicola@unb.br)

28  
29 Conflict of interest statement: The authors have declared that no conflict of interest exists.

30

## 31 Graphical abstract



32

33

## 34 Abstract

35 *Cryptococcus* spp. are important human pathogens responsible for about 180,000 deaths per year.  
36 Studying their virulence attributes can lead to better cryptococcosis prevention and treatment. In this  
37 work, we systematically investigated virulence attributes of *Cryptococcus* spp. clinical isolates and  
38 correlated them with patient data. We collected 66 *C. neoformans* and 19 *C. gattii* isolates from  
39 Brazilian patients and analyzed multiple phenotypes related with their capsule, production of laccase,  
40 melanin and extracellular vesicles. We also tested their virulence in *Galleria mellonella* and ability to  
41 evade macrophage LC3-associated phagocytosis (LAP). All phenotypes analyzed varied widely among  
42 the isolates, but *C. neoformans* isolates tended to melanize faster and more intensely and produce  
43 thinner capsules in comparison with *C. gattii*. We also observed correlations that match previous  
44 studies, such as that between secreted laccase – but not total melanin production – and disease outcome  
45 in patients. The most striking results, though, came from our measurements of *Cryptococcus* colony  
46 melanization kinetics, which followed a sigmoidal curve for most isolates. Faster melanization  
47 correlated positively with LAP evasion, virulence in *G. mellonella* and worse prognosis in humans.  
48 These results suggest that the speed of melanization, more than the total amount of melanin  
49 *Cryptococcus* spp. produces, is crucial for virulence.

## 50      **Introduction**

51              Cryptococcosis is estimated to cause 181.000 deaths per year, mostly in low and middle-  
52      income countries (1). Infection occurs through inhalation of spores or desiccated yeast cells of the  
53      species complexes *Cryptococcus neoformans* or *C. gattii*. In most cases, the disease happens in hosts  
54      with defective immunity secondary to AIDS, cancer or medication (2). The association of  
55      Amphotericin B and flucytosine is the gold standard treatment (3), but even in high income countries  
56      the case fatality rates are around 20%. In regions where these drugs are not available and fluconazole  
57      is the only choice, the case fatality rates can be higher than 60% (1). Combined with the fact that  
58      there are no vaccines, there is a clear need for more effective preventative and therapeutic options.

59              *C. neoformans* and *C. gattii* have several well-studied virulence factors such as the ability to  
60      produce melanin (4), the presence of a polysaccharide capsule (5), the ability to grow at 37 °C and the  
61      secretion of urease and other extracellular enzymes (6). Of these, the capsule and melanin contribute  
62      almost half of the *C. neoformans* virulence (7). The cryptococcal capsule is composed mainly of  
63      polysaccharides, such as glucuronoxylomannan (GXM). Its presence and thickness interfere with  
64      macrophage phagocytosis, and capsular polysaccharides interfere with the activation and  
65      differentiation of T cells (5, 8). Studies in mice and translational studies with cryptococcosis patient  
66      samples have shown the importance of GXM secretion by *C. neoformans* on central nervous system  
67      infections by *C. neoformans* (9, 10).

68              Melanin is a brown or black, hydrophobic, high molecular weight, negatively charged  
69      pigment. It is present in the cryptococcal cell wall and protects the yeast cell against host and  
70      environmental stresses (11). The pigment also increases resistance to Amphotericin B and affects  
71      susceptibility to fluconazole (12). In pathogenic species of the genus *Cryptococcus*, melanin  
72      production is dependent on laccase enzymatic action on biphenolic compounds (13). A translational

73 study with *C. neoformans* clinical isolates demonstrated that laccase also has other roles that are  
74 crucial for fungal survival in the cerebrospinal fluid, and that isolates with more effective melanin-  
75 independent secreted laccase roles were associated with poorer patient outcomes (14).

76 Given that several tools presently used to prevent and treat infectious diseases target  
77 microbial virulence factors, we delved further into the roles played by capsule, melanin, laccase and  
78 extracellular vesicles in the interaction between *Cryptococcus* spp. and their host. The strategy  
79 consisted of collecting clinical isolates and patient data, characterizing the isolates in the laboratory  
80 and correlating the experimental results with patient outcomes. We found several correlations that  
81 confirm previous observations, but also important correlations between the melanization kinetics and  
82 outcomes of the interaction between *Cryptococcus* spp. and macrophages or *G. mellonella*. Most  
83 importantly, we also found that faster melanization, but not the final amount of melanin on  
84 cryptococcal colonies, correlated with the survival of HIV-positive patients with severe  
85 cryptococcosis. These findings help us better understand the mechanisms used by *Cryptococcus* spp.  
86 to survive and cause disease in their hosts.

87 **Results**

88 **Clinical and epidemiological data**

89 *Cryptococcus* spp. clinical isolates analyzed in this study are from two different sources.

90 Twenty-eight (16 *C. neoformans* and 12 *C. gattii*) are from a cohort of patients treated in Rio de

91 Janeiro, Brazil, about whom we have no clinical information. The second group of clinical isolates

92 are from an ongoing epidemiological study in Brasília, Brazil. It contains 54 isolates (7 *C. gattii* and

93 47 *C. neoformans*) from 41 patients. From these, 37 were from the cerebrospinal fluid (CSF), 1 from

94 a blood culture, 1 from a tissue biopsy and 2 from bronchoalveolar lavage fluid. Thirty-seven patients

95 were infected by *C. neoformans* only, 2 by *C. gattii* only and the other 2 had *C. neoformans/C. gattii*

96 mixed infections. Available information about them and the patients from which they were isolated

97 can be found in Table S1.

98 All patients from the Brasília study (Table 1) were diagnosed and treated in public hospitals,

99 according to the standards used by the services in which they were assisted. Most were male (68.3%),

100 and their mean age was 42 years. HIV infection was reported in 68.3%; of the 12 HIV-negative

101 patients, two had diabetes, three were using corticosteroids, one was using corticosteroids plus a

102 second immunosuppressive drug, one had a primary immunodeficiency and eight had no known risk

103 factor. Among the 39 patients that we were able to follow until death or hospital discharge, the 2-

104 week and 10-week mortality rates were respectively 30.8% and 41%.

105 **Melanization kinetics**

106 The method we used to measure melanin production by each isolate quantitatively was based

107 on a previously published protocol (15) (Figure S1A). To do so, we spotted a specific number of

108 fungal cells in 24-well plates filled with solid medium containing the melanin precursor L-DOPA.

109 These plates were photographed at regular intervals during incubation, and the resulting digital

110 images processed to quantify how dark the colonies had become at each point in time. For most  
111 isolates, the resulting data fit very well in a sigmoidal curve. This method was highly reproducible, as  
112 shown by the similarity between the curves obtained from five independent experiments performed  
113 in different days with the control strain H99 (Figure S1B). As shown in Figures S1C-D, some  
114 isolates, such as H99, melanized faster and became black at the end of the experiment, whereas  
115 others, such as CNB017.1, melanized slowly and never became black. We also found differences in  
116 the pattern of colony melanization, such that some isolates (e.g. H99 and CNB017.1) showed  
117 homogeneously pigmented colonies whereas others had more intense melanization either in the  
118 periphery (CNB013.1) or in the center (CGF007) of the colony.

119         Using logistic regression, we quantitatively evaluated the kinetics of melanin production by  
120 each isolate. This regression resulted in five melanization parameters, three of which with a specific  
121 biological meaning:

122         • Bottom – median gray level of the colony at the first time point.

123         • Top – median gray level of the colony at the end of the experiment. Indicates how dark the  
124                 colony becomes, and thus the final amount of melanin it produces.

125         • Span: Top minus Bottom.

126         • Hill Slope: steepness of the curve. Indicates how fast the colony produces melanin during the  
127                 time in which melanization is occurring.

128         • EC50: Time it takes for the colony to reach half of its final melanization intensity. Measures both  
129                 how soon the colony starts melanizing and how fast it produces melanin once it has started.

130         A great variety was observed in the melanization parameters between isolates (Figure 1A-C),  
131 except for the melanization slope, which has a less dispersed frequency distribution (Figure 1D). To  
132 validate this new methodology, we compared its results with a semi-quantitative analysis we had

133 previously done of 16 clinical isolates (Figure S2A). These isolates were grown in solid melanin-  
134 inducing medium and photographed every 12 h for 7 days. After cropping all photos of each colony  
135 together, we visually ranked them based on how fast they melanized and how dark they eventually  
136 became. The isolates were then given a score of 1 to 7: 1 for those with slowest melanization and less  
137 dark colonies and 7 for those with the highest rate of colony pigmentation. We found a significant  
138 correlation between this visual score and the logistic regression parameter Top (Figure S2B),  
139 indicating that our image analysis method matches the visual ranking. As expected, the visual  
140 melanization score also correlated directly with Span ( $r = 0.456$ ) and Hill Slope ( $r = 0.226$ ) and  
141 inversely with EC50 ( $r = -0.254$ ), but these correlations were not statistically significant (p-values of  
142 0.066, 0.379 and 0.321, respectively).

143 In addition to melanization, we also measured laccase activity both on washed whole cells  
144 ( $n = 84$ ) and on the culture supernatants ( $n = 82$ ). We observed greater dispersion in the frequency  
145 distribution of secreted laccase activity (Figure 1E) than whole-cell laccase activity (Figure 1F).  
146 Interestingly, the laccase activity in culture supernatants, but not on whole cells, correlated well with  
147 the visual melanization score (Figure S2C) and melanization EC50 (Figure S2D).

148 **Clinical isolates demonstrate the variation of secreted GXM and capsule size in different  
149 culture media**

150 To evaluate the capsule from each clinical isolate, we grew them in different media,  
151 photographed the cells with India ink and measured their capsule thickness. The media we used  
152 included Sabouraud, a rich medium in which the cryptococcal capsule is not induced, and three capsule  
153 inducing media: Sabouraud diluted 1:10 with MOPS pH 7.5 (Sab-MOPS), minimum medium (MM)  
154 and CO<sub>2</sub>-independent medium (CIM). The baseline capsule thickness in non-inducing medium varied  
155 from 0.5 to 5  $\mu$ m for different isolates, although more than 90% of the clinical isolates had capsules 1

156 to 2  $\mu$ m thick (Figure 2A). Representative pictures of clinical isolates at both extremes variation of  
157 capsule thickness is shown in Figure 2B. To determine the capacity for capsule induction, we measured  
158 the capsules for each isolate in each inducing medium and divided the value by that obtained in  
159 Sabouraud (Figure 2C-F). Sab-MOPS medium resulted in the greatest capsule induction, the most  
160 notable of which that of CNF016 (Figure 2 D). However, even in this medium some isolates such as  
161 CGB009.1 maintained a capsule thickness that was very similar to that in Sabouraud, indicating that  
162 different isolates may respond differently to the signals that induce capsule.

163 As capsular polysaccharides can be secreted in soluble form, we also used a capture ELISA to  
164 determine the concentrations of GXM on the culture supernatants of the clinical isolates. As observed  
165 with other virulence factors, the values we obtained varied widely across clinical isolates (Figure 2G).

166 **Clinical isolates present different profiles of interaction with macrophages**

167 Macrophages are crucial effector cells in the immune response to *Cryptococcus* spp. A specific  
168 type of autophagy, LC3 associated-phagocytosis (LAP), is important for the fungicidal activity of  
169 macrophages (16). As previous studies with *Aspergillus fumigatus* showed that melanin inhibited LAP  
170 (17, 18), which is important in immunity against *C. neoformans* (19), we quantified LAP in  
171 macrophage-like J774 cells infected with antibody-opsonized clinical isolates.

172 The infected J774 cells were processed for LC3 immunofluorescence microscopy and imaged.  
173 For each isolate, we evaluated the images to calculate two variables: the proportion of macrophages  
174 with LC3 recruitment to at least one phagosome containing *C. neoformans* and the proportion of  
175 macrophages where all internalized fungi were on LC3-positive phagosomes. Figure 3A shows  
176 representative immunofluorescence images with the two isolates that had the lowest (CNB020) and the  
177 highest (CNB042) proportion of LC3-positive phagosomes. In none of the isolates tested all

178 internalized fungi were noticed in LC3-positive phagosomes (Figure 3B), possibly indicating that  
179 *Cryptococcus* spp. has mechanisms to avoid this type of macrophage response.

180 ***C gattii* isolates have larger capsules and more secreted laccase activity, but *C. neoformans***  
181 **melanizes faster and more intensely**

182 After systematically measuring virulence and host-pathogen interaction attributes, we began  
183 mining them for important insights into cryptococcal virulence. We observed important differences in  
184 the expression of virulence factors between *C. neoformans* and *C. gattii*. A larger proportion of *C.*  
185 *neoformans* isolates (23 out of 57) had a homogeneous colony melanization pattern, in comparison  
186 with just one out of 19 *C. gattii* isolates ( $p = 0.004$ , Fisher's exact test). *C. neoformans* isolates also  
187 melanized faster (lower melanization EC50) and accumulated more melanin at the end of the  
188 experiment (higher melanization Top), with no differences on Hill Slope and Span (Figure 4 A-D). The  
189 two species also differed in laccase activity, with more secreted laccase activity in the supernatants –  
190 but not whole cells – of *C. gattii* isolates (Figure 4 E-F).

191 *C gattii* isolates had thicker capsules than *C. neoformans* in non-inducing Sabouraud medium  
192 (Figure 4G). *C gattii* isolates also induced their capsules to a larger extent than *C. neoformans* in all  
193 capsule-inducing media, with the differences being statistically significant in all but minimal medium  
194 (Figure 4H-J). The slightly higher amount of GXM secreted into the supernatant of *C. gattii* cultures  
195 was not significant ( $p = 0.319$ , two-tailed t-test) (data not shown).

196 **The amount of secreted extracellular vesicles correlates with capsule thickness, melanization**  
197 **and secreted laccase activity**

198 Given that extracellular vesicles (EVs) are necessary for the export of capsular  
199 polysaccharides and laccase (20, 21), we also studied the EVs isolated from a subset of *C.*  
200 *neoformans* isolates. We used an indirect method to quantify them, measuring the concentration of

201 ergosterol in cell-free supernatants (22) and dividing this by the number of cells (Figure 5A).  
202 Besides, we also measured the hydrodynamic diameters and polydispersity indices of the vesicle  
203 preparations by dynamic light scattering (DLS) (Figure 5B-C). We observed significant correlations  
204 between EV-ergosterol content and the visual melanization score and melanization EC50 (Figure 5D-  
205 E), but not with melanization Top and Span (Figure 5F-G). EV-ergosterol also showed significant  
206 correlation with whole-cell laccase activity and secreted laccase activity (Figure 5H-I). The indirect  
207 EV measurement in the supernatants of clinical isolates also correlated well with their basal capsule  
208 thickness in Sabouraud, but not with their ability to induce capsule in any of the three tested media  
209 (Figure 5J-N).

210 **Melanization kinetics of clinical isolates affect the ability to escape from LC3- associated  
211 phagocytosis in macrophages**

212 We correlated the LAP proportions described above with melanin and capsule phenotypes.  
213 The proportion of macrophages with LC3-positive phagosomes correlated strongly with melanization  
214 EC50 and inversely with melanization Top and Span (Figure 6A-C), but not with secreted laccase  
215 activity (Figure 6D). In contrast, no significant correlation between the proportion of macrophages  
216 with LC3-positive phagosomes and any of the capsule variables was found (Figure S3).

217 **Melanization kinetics, laccase activity and the capsule of clinical isolates affect survival in *G.  
218 mellonella***

219 We infected wax moth (*G. mellonella*) larvae with the clinical isolates to evaluate the role of  
220 melanization, laccase activity and capsule on virulence. This experiment was made with 15 *C. gattii*  
221 and 31 *C. neoformans* isolates divided into three lots. The survival curves for one of these lots is  
222 shown as an example in Figure 7A, whereas the distribution of median *G. mellonella* larvae survival  
223 times is shown in Figure 7B.

224         Using Cox proportional hazards regression, a multivariate survival tool, we generated a  
225         statistical model which evaluated the impact of virulence phenotypes upon survival time for all  
226         infected larvae (n = 588). In addition to virulence variables, we added as possible confounding  
227         variables the *Cryptococcus* species of the isolate and diet fed to *G. mellonella* larvae used for  
228         infections. The Cox regression model ( $X^2 = 258.9$ , df = 10, p < 0.001) showed significant impacts on  
229         *G. mellonella* survival of capsule induction in Sab-MOPS (HR 1.7, 95% CI 1.2–2.4, P = 0.003),  
230         melanization EC50 (HR 0.8, 95% CI 0.7–0.9, P = 0.02), melanization Top (HR 2.9, 95% CI 1.6–5.2,  
231         P < 0.001), secreted laccase activity (HR 2.6, 95% CI 2.0–3.3, P < 0.001) and the confounding co-  
232         variable cereal diet (HR 7.5, 95% CI 5.0–11.2, P < 0.001). Variables that were not significant on this  
233         Cox regression model are capsule thickness in Sabouraud, capsule induction in MM and CIM, whole-  
234         cell laccase activity and species of *Cryptococcus*.

235         **Secreted laccase activity and melanization EC50 increases the risk of death in patients with**  
236         **disseminated cryptococcosis**

237         To study the impact of these phenotypes on the human disease, we used survival data from  
238         patients of the Brasília study for a Cox proportional hazards regression. Of the 41 patients, we  
239         included in the regression model only those with systemic disease caused by *C. neoformans* or mixed  
240         *C. neoformans/C. gattii* (n = 34). Eight patients were HIV negative and 23 HIV positive. The  
241         maximum follow-up time was 303 days. Patients who died during the follow-up time were accounted  
242         as events (n = 19), whereas the other ones were released after treatment (n = 14) or lost to follow up  
243         while still alive (n = 1). The Cox regression model ( $X^2 = 13.7$ , df = 6, p = 0.032) showed significant  
244         impacts on patient survival of the melanization EC50 (HR 0.23, 95% CI 0.06–0.83, P = 0.025) and  
245         secreted laccase activity (HR 5.07, 95% CI 1.22–21.09, P = 0.025). Covariates that were not  
246         significant on the Cox regression model were age at the time of diagnosis, HIV status, whole-cell  
247         laccase activity and melanization Top.

## 248     Discussion

249           Capsule and melanin are two of the most important and studied virulence factors in the  
250           *Cryptococcus* genus, allowing the fungal cells to subvert host immunity and cause cryptococcosis.  
251           Most studies on these virulence factors are made either in vitro or in animal models, which are both  
252           highly informative but not completely applicable to human disease. We used a translational approach  
253           to study virulence phenotypes in clinical isolates, their interaction with experimental hosts and finally  
254           to associate them with the outcome of the disease in the patients from whom the isolates were  
255           obtained. We were thus able to obtain novel information on the role these two virulence attributes  
256           play in the pathogenesis of cryptococcal disease.

257           The most striking observations were related to melanin, a pigment that protects cryptococcal  
258           cells against oxidative stress, extreme temperatures and UV radiation (23–27). All isolates tested  
259           were able to produce the pigment, although some produced it faster than others and appeared darker  
260           at the end of the experiment. This melanization speed correlated negatively with the survival of  
261           cryptococcosis patients, suggesting a poor prognosis in infections caused by fast melanin forming  
262           strains. Sabiiti and colleagues (14) have previously shown that the amount of secreted laccase  
263           correlated well with cryptococcal survival in the cerebrospinal fluid and patient outcome, but when  
264           they analyzed the amount of melanin made, they were unable to establish a statistically significant  
265           correlation at the  $p = 0.05$  level, which was possibly at Type II error. What they measured, however,  
266           was the total amount of melanin in the cells of a subset of ten clinical isolates, which might be similar  
267           to the Melanization Top variable we measured. Our observations suggest that the melanization speed,  
268           rather than the final amount of melanin, could be more important in determining the outcome of  
269           human cryptococcosis. This observation makes sense given that melanization protects cells against  
270           immune mechanisms and cells that melanize earlier would have a survival advantage.

271 Because laccase synthesizes melanin, melanization parameters are expected to depend to  
272 some extent on laccase production, which means that teasing their roles on outcomes of infection  
273 apart can be challenging. However, the present study suggests these roles are at least partly  
274 independent. Specifically, figure S2D shows that while laccase secretion generally correlates with  
275 melanization speed, outliers do exist that either secrete relatively large amounts of the enzyme, but  
276 melanize slowly, and melanize fast while secreting relatively low amounts of the enzyme.  
277 Differences in the efficiency of melanin anchoring in the cell wall, in the proportion of free and cell  
278 wall-associated laccase, or in the proportion of chitin in the cell wall (the polymer to which melanin  
279 is anchored (28)) may perturb the correlation between the two measurements. Statistically, residual  
280 plots of our correlation data are symmetric around zero for both variables, which also suggests the  
281 assumptions of our model are correct. Moreover, laccase has well-documented effects in infection  
282 outcomes that are melanin-independent, such as detoxification of iron (29), prostaglandin production  
283 (30) and neutralization of the fungicidal properties of cerebrospinal fluid (14).

284 Extracellular vesicles (EVs) are associated with several biological roles (31). In fungi,  
285 especially *Cryptococcus*, they are associated with the transport of various important virulence  
286 molecules like melanin, laccase, nucleic acids and others (20, 21). A *C. neoformans* mutant with  
287 impaired EV secretion, obtained through silencing of the *SEC6* gene, presented a decrease in secreted  
288 laccase activity and was hypovirulent in mice (20). The formation of capsule and melanin are  
289 dependent on the secretion of EVs (32, 33). Hence, we quantified the association between EVs and  
290 the phenotypes of the capsule, laccase and melanin of a subset of the isolates, all of the same species  
291 of *C. neoformans* and molecular type VNI. The amount of EVs secreted in minimal medium had a  
292 strong correlation with laccase activity. Interestingly, we also found that the amount of EVs are  
293 associated with faster melanization and the larger capsule thickness in rich medium. On the other  
294 hand, we found no correlation with the total amount of melanin, the total amount of secreted GXM or

295 the thickness of the capsule in nutrient-deficient inducing media. These findings highlight the role of  
296 EVs in the expression of *C. neoformans* virulence factors. Taken together, our data indicate that  
297 factors other than the amount of EVs are important for inducing capsule in nutrient-deficient media.

298 Melanization phenotypes and laccase activity are associated with greater virulence of clinical  
299 *Cryptococcus* spp. Isolates (8, 14, 34–38). Genetic and environmental factors contribute to the  
300 variation in the melanin production of *C. neoformans* (39, 40), which is under complex cellular  
301 regulation (40, 41). Furthermore, the experimental quantification of the pigment presents significant  
302 methodological challenges (42). *C. neoformans* showed better melanization capacity compared to *C.*  
303 *gattii*. But interestingly, *C. gattii* showed a greater capsule thickness in all media, except for minimal  
304 medium (which mimics the environment of cryptococcal meningitis infection, the main manifestation  
305 in most severe cases of cryptococcosis). We found that isolates with higher basal capsule thickness  
306 (Sabouraud) had a significant correlation with isolates with lower melanization speed (high  
307 melanization EC50), but this melanization index did not correlate with capsule thickness in MM. We  
308 found significant correlations between secreted laccase activity and relative capsule thickness in Sab-  
309 MOPS /Sabouraud medium after 24h of culture. Overall, these data suggest that melanization  
310 kinetics, secreted laccase activity and capsule thickness have different expression mechanisms  
311 between species. In addition, laccase activity differs not only between species but also at the cellular  
312 location where it is quantified. Perhaps higher melanin production and lower capsule thickness are  
313 important factors that favor the dissemination and survival of *C. neoformans* in the central nervous  
314 system, unlike *C. gattii*, which is strongly associated with pulmonary cryptococcosis (43).

315 The evolutionary divergence of proteins and signaling cascade configurations between *C.*  
316 *neoformans* and *C. gattii* may explain the differences in the expression of virulence factors. For  
317 example, TPS1 and TPS2 genes were found to be critical for thermotolerance, pathogenicity, capsule  
318 and melanin production in *C. gattii* (44), but the homologous genes in *C. neoformans* were required

319 only for thermotolerance, not for capsule or melanin production (45). This may indicate that the  
320 expression of the capsule is influenced not only by the yeast environment, but also by different  
321 genetic traits between the two species (10). Here we hypothesize that *C. gattii* ability to infect  
322 immunocompetent individuals can be partly explained by the increased basal expression of important  
323 virulence factors such as the polysaccharide capsule.

324 Macrophages are crucial effector cells against fungi. However, some facultative intracellular  
325 pathogens, like *Cryptococcus* spp., can survive and replicate inside macrophages. These cells trigger  
326 autophagy as part of their response to intracellular pathogens (46, 47). The presence of LC3  
327 (microtubule-associated protein 1 light chain 3 alpha) is associated with autophagosome maturation  
328 (48). The autophagy route called LC3 associated-phagocytosis (LAP) is important for the fungicidal  
329 activity of macrophages (16). Fungal virulence factors, such as melanin in *Aspergillus fumigatus*,  
330 inhibited LAP and increased virulence, *in vitro* and in a murine model (17, 18). In a subset of the  
331 clinical isolates, we observed a strong correlation between the melanization kinetics and the  
332 inhibition of LAP in murine macrophages.

333 Wax moth larvae (Lepidoptera) are an invertebrate animal model extensively used for *in vivo*  
334 studies of *Cryptococcus* virulence, host innate immunity after infection and the activity of antifungal  
335 compounds (49–51). Bouklas and collaborators showed that intracellular phagocytosis, killing by  
336 murine macrophages, capsule thickness and laccase activity did not correlate with *C. neoformans*  
337 virulence in *G. mellonella*. They found high-uptake strains to have significantly increased laccase  
338 activity, and virulence in mice, but not in *G. mellonella* (52). It should be added that methodological  
339 differences might explain the discrepancy in observations: Bouklas *et al.* measured laccase activity  
340 by melanin accumulation in a liquid culture over 16 hours at 37 °C, plus 24 hours at 25 °C, which  
341 corresponds roughly to the whole-cell laccase activity quantitation protocol we used, whereas we  
342 measured secreted laccase separately. In other words, there is no disagreement between our data and

343 theirs, since in our work whole-cell laccase also had no detectable influence on the outcome of  
344 *Galleria* infection. Another study found that *C. gattii* strains exhibited similar virulence between  
345 murine inhalation models and *G. mellonella* infection (53). Following evidence of a deterministic  
346 system in *G. mellonella* cryptococcal infection (54), our results support the idea that virulence is an  
347 emerging property that cannot be easily predicted by a reductionistic approach, but can be partially  
348 resolved by the multivariate regression model. Furthermore, our result is in agreement with a  
349 previous report (51) that melanin synthesis was directly related with the level of virulence of four  
350 major molecular types of *C. gattii* in *G. mellonella*.

351 In summary, our study showed that melanization kinetics, secreted laccase activity and capsule  
352 growth in different inducing media are each associated with the virulence of clinical *Cryptococcus*  
353 strains in an invertebrate animal model. EVs, laccase secretion and melanin production represent a  
354 continuum that seems to exert the major influence on infection outcomes. A limitation of this study is  
355 that our patients were treated with different regimens in distinct health services. In our cohort of  
356 patients, faster colony pigmentation together with secreted laccase activity had a significant  
357 association with mortality attributed to disseminated cryptococcosis. The clinical isolates *C.*  
358 *neoformans* CNB004.1, CNB007.1 and CNB020 presented the lowest MelEC50 values of (faster  
359 melanization). Accordingly, they were found to be highly virulent in that the patients that harbored  
360 them all died within 25 days after diagnosis with cryptococcal meningoencephalitis. Altogether, we  
361 have seen strong correlations and trends, and although this does not necessarily imply causality, the  
362 strength and pattern of the associations indicate that melanization kinetics plays a key role in  
363 cryptococcal disease. These findings highlight the importance of the role of the laccase-dependent  
364 melanin pathway and its relevance to the human clinical outcomes, which suggests the EV-laccase-  
365 melanin nexus as an important source of targets for future therapeutic approaches to disseminated  
366 cryptococcosis.

367 **Methods**

368 **Patients and *Cryptococcus* spp isolates**

369 The isolates used in the study were obtained from two different sources in Brazil. One set came from  
370 the Culture Collection of Pathogenic Fungi at Fiocruz, in the city of Rio de Janeiro. The other one  
371 from an ongoing epidemiological study in the city Brasília. This study was approved by the Ethics  
372 Committee of the Foundation for Teaching and Research in Health Sciences (CEP-FEPECS).  
373 Patients or their legal guardians gave written informed consent for the collection of clinical data and  
374 specimens, including those from which fungal isolates were obtained.

375 A total of 82 clinical isolates were used. We added two control strains (H99 and B3501). The 28  
376 isolates from Rio de Janeiro had been previously typed by MLST as *C. neoformans* molecular type  
377 VNI and *C. gattii* molecular type VGII, whereas the isolates from Brasília were typed by Sanger-  
378 sequencing an URA5 amplicon and comparing the sequence to that of standard strains. With this  
379 analysis, 65 isolates were determined to belong to the *C. neoformans* species complex, molecular  
380 type VNI, and 19 were typed as belonging to the *C. gattii* species complex, molecular type VGII. We  
381 were able to obtain clinical information from the medical records of 41 patients, which are shown in  
382 Table 1.

383 ***Cryptococcus* culture**

384 All yeasts were kept frozen in 35% glycerol on a -80°C freezer. From this stock, the isolates were  
385 streaked onto Sabouraud agar medium (2% dextrose, 1% peptone, 1.5% agar, pH 5.5) and grown for  
386 72 hours at 30 °C. Isolated colonies were incubated for 72 hours in Sabouraud-dextrose (Sab) liquid  
387 medium (4% dextrose, 1% peptone, pH 5.5; Sigma-Aldrich) at 37°C with 250 rpm shaker rotation.  
388 Laboratory reference strains H99 (*C. neoformans* var. grubii, serotype A) and B3501 (*C. neoformans*  
389 var. neoformans, serotype D) were used as controls.

390 **Melanin production evaluation**

391 The minimal medium (MM) (15 mM d-Glucose, 10 mM MgSO<sub>4</sub>, 29.4 mM KH<sub>2</sub>PO<sub>4</sub>, 13 mM glycine,  
392 3 µM thiamine-HCl) (26) was used in these experiments. The 2X concentrated MM and 3% agar  
393 were prepared separately. As thiamine and L-DOPA can be degraded at high temperatures, the agar  
394 was heated separately and, after reaching the temperature of 60°C, was mixed with the 2X MM,  
395 reaching a final concentration of 1.5% agar and 1X MM. After that, the medium was supplemented  
396 with 1 mM L-DOPA (Sigma-Aldrich). Then 1 mL of the MM agar was added to each well in a 12  
397 well plate protected from light. In the center of each well, 5 µL of each isolate containing a total of  
398 10<sup>5</sup> cells were inoculated. All inocula were made in duplicates, in two different wells. The plates  
399 were incubated at 37 °C protected from light and monitored for 7 days. After 24 hours of incubation,  
400 we photographed each plate every 12 hours. The photographs were taken inside a biosafety cabinet,  
401 using a mirror apparatus specially prepared to photograph culture plates. With these conditions, it  
402 was possible to standardize the illumination and photography parameters during the capture of all  
403 images. A Nikon D90 digital single-lens reflex (D-SLR) camera equipped with and 85 mm lens was  
404 used, with fixed focal length, exposure time, ISO setting, aperture, and white balance. The colony  
405 melanization phenotype was also evaluated and classified into heterogeneous (melanization mostly at  
406 the center or edges of the colony) or homogeneous colony melanization.

407 **Semi-quantitative melanization score**

408 Based on the images collected as described above, the isolates were qualitatively categorized into 7  
409 groups ordered from 1 to 7. Group 1 contains the colony with less intense and slower melanization  
410 and group 7 the isolates with more intense and faster melanin production. Each isolate was scored  
411 independently by two researchers that were blinded to each other's evaluation, and the outcomes  
412 were very similar.

413 **Melanin quantification in grayscale**

414 We also created a protocol based on previous publications (15, 55) to quantitatively analyze the  
415 images described above. All images were manipulated using Adobe Photoshop CS6 and ImageJ  
416 version 2.0.0-rc-65 / 1.52a. No nonlinear modifications were done on the original images. The photos  
417 were adjusted in Photoshop to show each plate horizontally and with a size of 2400 x 1800 pixels.  
418 Each image was then exported to ImageJ, converted to 8-bit gray scale and inverted. The "ROI" tool  
419 was used to measure the colonies to obtain the median, mean and area values. The median gray level  
420 values were the averaged for the two colonies from each isolate. These values were fit to a nonlinear  
421 regression equation [Agonist] vs. response - Variable slope (four parameters) on GraphPad Prism.  
422 Strain H99 was used as an internal control in each experiment. All values were normalized by the  
423 value obtained with H99 in the respective experiment.

424 **Laccase activity**

425 Each strain was inoculated into 5 mL YPD (2% glucose, 2% yeast extract) and incubated with 200  
426 rpm agitation at 30°C for 24 hours. The cells were harvested by centrifugation at 1,000 × g for 10  
427 minutes and the culture was resuspended and incubated at 30°C, for 5 days, in 5 mL of asparagine  
428 salts medium with glucose (0.3% glucose, 0.1% L-asparagine, 0.05% MgSO<sub>4</sub>, 1% of solution 1 M  
429 (pH6.5) Na<sub>2</sub>HPO<sub>4</sub>, 3 µM thiamine, 0.001% of solution 0.5 M CuSO<sub>4</sub>). The cells were harvested by  
430 centrifugation at 1,000 × g for 10 minutes and washed once with 5 mL 50 mM Na<sub>2</sub>HPO<sub>4</sub> pH 7.0 and  
431 washed once with 3 mL asparagine salts medium without glucose. Yeast cells were counted in a  
432 hemocytometer and adjusted to achieve an inoculum 10<sup>8</sup> cells/mL. The same number of cells were  
433 resuspended in 5 mL of the medium without glucose and incubated at 30°C for 72 hours to induce  
434 laccase expression. After incubation, the number of yeast cells per sample were counted in a  
435 hemocytometer to normalize the secreted laccase activity in the supernatant by the number of cells

436 present at the end of the culture. The supernatants were harvested at 4,000 g for 5 minutes, and  
437 secreted laccase activity was measured in a 96-well plate by adding 180  $\mu$ L of supernatant from each  
438 isolate and 20  $\mu$ L of 10 mM L-DOPA. To measure laccase activity in whole cells, we prepared  
439 suspensions of  $10^8$  cells/mL of each isolate. In 24-well plates, we placed 450  $\mu$ L of each isolate  
440 suspension plus 50  $\mu$ l 10 mM L-DOPA. In 96-well plates, we placed 180  $\mu$ L of the yeast suspension  
441 and 20  $\mu$ l 10 mM L-DOPA. The blank for laccase activity in living cells was 500 and 200  $\mu$ L cell  
442 suspension, respectively. The amount of pigment produced was determined spectrophotometrically at  
443 a 480-nm wavelength read every hour for 6 to 48 hours. Absorbance values (OD) converted into  
444 laccase activity per yeast with the equation: laccase activity ( $\mu$ mol/number yeast) = mean of OD/(7.9  
445 x number yeast at the end of culture). For L-DOPA we used a standard molar extinction coefficient  
446 of 7.9  $\mu$ mol<sup>-1</sup> (56, 57). Results were reported as the mean of two or three experiments. To adjust for  
447 interexperimental variation, laccase activity of the clinical isolates was expressed as a ratio to H99  
448 (positive control).

#### 449 **ELISA for GXM quantitation secreted in supernatant**

450 Inocula containing  $10^5$  cells/mL of each isolate were incubated in 3 mL of minimal medium for 72  
451 hours at 30°C under 200 rpm shaking. The cells were precipitated by centrifugation at 4,000  $\times$  g for  
452 10 minutes, 4°C and 1.5 mL of the supernatant was transferred to microcentrifuge tubes. Cells debris  
453 were precipitated by centrifugation at 15,000  $\times$  g for 30 minutes, 4°C and 1 mL of the supernatant  
454 was filtered through a 0.45  $\mu$ m syringe filter (polycarbonate membrane) and kept at -20 ° C until the  
455 time of the ELISA assay. The supernatants were diluted 5,000 and 10,000-fold and analyzed for  
456 GXM by enzyme-linked immunosorbent assay (ELISA). A 96-well high binding polystyrene plate  
457 (Costar, #25801) was coated for 1 hour at 37°C with supernatants samples and standard curve of  
458 GXM from 0 to 10  $\mu$ g/mL (H99 GXM standard) and then blocked with 1% bovine serum albumin for  
459 1 hour at 37°C. The plates were then washed three times with a solution of Tris-buffered saline

460 (0.05%Tween 20 in PBS), followed by the detection of GXM with 50  $\mu$ L of the monoclonal IgG1  
461 18B7 (1  $\mu$ g/ml) for 1 hour at 37°C. The plates were washed three times again and binding of 18B7  
462 was detected with 50  $\mu$ L of alkaline phosphatase-conjugated GAM IgG1 (Fisher) (1  $\mu$ g/mL) for 1  
463 hour at 37°C. The plates were washed three more times and developed with 50  $\mu$ L of p-nitrophenyl  
464 phosphate disodium hexahydrate (Pierce, Rockford) (1 mg/mL). The absorbance was measured at  
465 405 nm after 15 minutes in an EON Microplate Spectrophotometer (Biotek Inc). After subtraction of  
466 the blank values, sample measurements were interpolated with a standard four-parameter sigmoidal  
467 curve. Values represent the averages of three independent culture and ELISA experiments, performed  
468 in different days. The result of each clinical isolate was normalized by the number of cells at the end  
469 of the culture time.

470 **Capsule formation**

471 Inocula were made from cultures grown overnight in Sabouraud medium. These cells were grown in  
472 24-well culture plates with four distinct liquid media, with a starting density of  $10^6$  yeasts/mL. A  
473 non-capsule-inducing medium (Sabouraud dextrose - Sab) and three capsule-inducing media (58, 59)  
474 were used: minimal medium (MM), ten-fold diluted Sabouraud in 50 mM MOPS (SabMOPS) and  
475 CO<sub>2</sub>-independent medium (Thermo Fisher Scientific) (CIM) for 24 hours at 37°C. Afterwards, 10  $\mu$ L  
476 of yeasts cells were stained using 1:1 India ink. The slides were photographed in a Zeiss Z1 Axio  
477 Observer inverted microscope using a 40X objective (EC Plan Neofluar 40X/0.75 Ph 2; Carl Zeiss  
478 GmbH) and an MRm cooled CCD camera (Carl Zeiss GmbH). Images were collected and the  
479 capsules measured with the ZEN 2012 software. Capsule thickness measurements were normalized  
480 by the value obtained with H99 in each experiment. The expressed results are a mean of three  
481 independent experiments performed on different days, with 20 cells measured per experiment.

482 **Analysis of extracellular vesicles (EVs)**

483 EVs were obtained from the culture supernatants of each clinical isolate as previously outlined (32),  
484 with modifications. Briefly, fungal cells were cultivated in 40 mL of minimal medium for 3 days at  
485 30°C with shaking. The cultures were then sequentially centrifuged to remove smaller debris, filtered  
486 through a 0.8 µm filter and the supernatants ultra-centrifuged (Beckman Coulter optima I-90k, SW28  
487 rotor). The precipitate was resuspended in 2 mL of the remaining culture medium. With 1 mL of EV  
488 preparations, the hydrodynamic diameter (intensity) and the polydispersity index were measured by  
489 Dynamic Light Scattering (DLS) (ZetaSizer Nano ZS90 (Malvern,UK). Vesicle quantification was  
490 performed based on the analysis of sterol in their membranes, using a quantitative fluorimetric  
491 Amplex Red sterol assay kit (Invitrogen, catalog number A12216), according to the manufacturer's  
492 instructions. All samples were analyzed in duplicate and under the same conditions.

493 **Interaction with macrophages from immunofluorescence microscopy and LC3-associated  
494 phagocytosis (LAP)**

495 The J774.16 cell line (J774) was purchased from the American Type Culture Collection (ATCC) to  
496 study the interaction of clinical isolates with macrophages. Cells were maintained at 37°C in the  
497 presence of 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10%  
498 heat-inactivated fetal calf serum (FCS) and 1% penicillin–streptomycin (fresh medium) (all from  
499 Invitrogen). Cells were used between 10 and 35 passages. J774 cells (2 x 10<sup>5</sup> cells) were plated in a  
500 13 mm round glass coverslip (previously treated with 5% HCL and heated to 90°C for 10 minutes)  
501 placed inside a flat-bottom 24-well tissue culture plate (Kasvi) and allowed to adhere for 24 hours.  
502 The cell monolayers were then infected with each IgG1-opsonized (mAb 18B7, 10µg/mL) clinical  
503 isolate in a proportion of two fungi per macrophage. The cells were co-incubated for 12 hours and  
504 then fixed and permeabilized with methanol at -20°C for 10 minutes. The cells were then incubated  
505 with rabbit polyclonal antibody to LC3 (Rabbit IgG anti-LC3-beta, Santa Cruz Biotechnology)  
506 followed by a fluorescein-conjugated secondary (Goat anti-Rabbit IgG conjugated Alexa Fluor 488,

507 Invitrogen). After staining, the coverslips were sealed in ProLong Gold Antifade (Invitrogen).  
508 Images were collected on a Zeiss Z1 Axio Observer inverted microscope at 63X (Plan-Apochromatic  
509 63X/1.4 NA, Carl Zeiss GmbH) and an MRm cooled CCD camera (Carl Zeiss GmbH) using the ZEN  
510 Blue 2 software. For each isolated, 100 macrophages with internalized *C. neoformans* cells were  
511 counted and scored as positive for LAP if at least one of the phagocytosed fungi was in an LC3-  
512 positive vacuole.

513 ***G. mellonella* median survival time**

514 *G. mellonella* larvae were reared in glass jars, at 30 °C in darkness. To maintain the colony, sufficient  
515 amounts of an artificial diet were added to the jars at least three times a week. Last instar larvae in the  
516 200 mg weight range were injected in the terminal left proleg with 10<sup>4</sup> yeast cells in ten microliters of  
517 PBS containing ampicillin at 400 µg/mL. Twelve individuals were infected per group and the larvae  
518 were kept at 37°C after infection. Deaths were counted daily and the experiment was terminated  
519 when all individuals in the PBS group molted. Molted individuals in any group were censored from  
520 the analysis at the day of their molting. The median survival for each clinical isolate was normalized  
521 with that observed for H99 in each experiment.

522 Negative control (inoculated with PBS) and positive control (inoculated with H99) groups were  
523 repeated in each experimental set. Two of the three sets were made with *G. mellonella* larvae that had  
524 been fed a cereal-based diet, whereas the third set with 11 clinical isolates were fed beeswax and  
525 pollen.

526 **Statistics**

527 Values of p lower than 0.05 were considered significant. Spearman's correlation was used to  
528 determine the correlation between *in vitro* phenotypes and *in vivo* patient outcomes. Differences  
529 between groups were determined using the two-tailed t-test for normally distributed data. Survival in

530 *G. mellonella* infection studies was obtained by log-rank (Mantel-Cox) test using GraphPad Prism  
531 software. Results from *G. mellonella* survival studies and were evaluated with Cox proportional  
532 hazards regression using IBM SPSS software.

533 **Study approval**

534 Clinical cryptococcal isolates used in this study were obtained from patients enrolled in a  
535 clinical study in Brazil. All trials were approved by the Ethics Committee of Fiocruz, Brazil and by  
536 the Research Ethics Committee of - FEPESCS/SES-DF, Brazil, number: 882,291 and 942,633.  
537 Patients or their legal guardians gave written informed consent, including authorization for the  
538 storage and use of their clinical isolates for future research. All patients were monitored and treated  
539 free of charge by the Brazilian national health system.

## 540    **Author contributions**

541    Conceived and designed the experiments: HRS, AMN, PA. Performed the experiments: HRS, SOF,  
542    GPOJ, CPR, KMG, EMG. Analyzed the data: HRS, AMN. Supplied funding, assisted in data  
543    analysis and oversaw writing of the paper: MSSF, ISP, PA, HCP and AC. Obtaining and tracking  
544    clinical isolates and data: WFF, AFC, JLJ, VLPJ, LT and MSL. Wrote the first manuscript draft:  
545    HRS and AMN. The manuscript draft was revised and approved by all authors.

## 546 Acknowledgements

547 A.M.N was funded by FAP-DF awards 0193.001048/2015-0193.001561/2017 and the CNPq grant  
548 437484/2018-1. M.S.S.F was supported by FAP-DF/PRONEX award 193.001.533/2016. AC was  
549 supported by the National Institutes of Health grants 5R01A1033774, 5R37AI033142, and  
550 5T32A107506, and CTSA grants 1 ULI TR001073-01, 1 TLI 1 TR001072-01, and 1 KL2 TR001071  
551 from the National Center for Advancing Translational Sciences. The authors would like to thank  
552 Jhones Dias and Alicia Ombredane for experimental assistance, as well as Emma Camacho and  
553 Radamés Cordero for the critical reading of the manuscript.

554 **References**

555 1. Rajasingham R et al. Global burden of disease of HIV-associated cryptococcal meningitis: an  
556 updated analysis. *Lancet Infect. Dis.* 2017;17(8):873–881.

557 2. Kwon-Chung KJ et al. The case for adopting the “species complex” nomenclature for the etiologic  
558 agents of cryptococcosis. *mSphere* 2017;2(1):1–7.

559 3. Perfect JR et al. Clinical practice guidelines for the management of cryptococcal disease: 2010  
560 update by the infectious diseases society of America. *Clin. Infect. Dis.* 2010;50(3):291–322.

561 4. Lin X, Heitman J. The biology of the *Cryptococcus neoformans* species complex. *Annu. Rev.*  
562 *Microbiol.* 2006;60(1):69–105.

563 5. Chang YC, Kwon-Chung KJ. Complementation of a capsule-deficient mutation of *Cryptococcus*  
564 *neoformans* restores its virulence. *Mol. Cell. Biol.* 1994;14(7):4912–4919.

565 6. Kozubowski L, Lee SC, Heitman J. Signalling pathways in the pathogenesis of *Cryptococcus*.  
566 *Cell. Microbiol.* 2009;11(3):370–380.

567 7. McClelland EE, Bernhardt P, Casadevall A. Estimating the relative contributions of virulence  
568 factors for pathogenic microbes. *Infect. Immun.* 2006;74(3):1500–1504.

569 8. Zaragoza O. Basic principles of the virulence of *Cryptococcus*. *Virulence* 2019;10(1):490–501.

570 9. Robertson EJ et al. *Cryptococcus neoformans* ex vivo capsule size is associated with intracranial  
571 pressure and host immune response in hiv-associated cryptococcal meningitis. *J. Infect. Dis.*  
572 2014;209(1):74–82.

573 10. Denham ST et al. Regulated release of cryptococcal polysaccharide drives virulence and

574 suppresses immune cell infiltration into the central nervous system. *Infect. Immun.* 2018;86(3).

575 doi:10.1128/IAI.00662-17

576 11. Nosanchuk JD, Casadevall A. Impact of melanin on microbial virulence and clinical resistance to

577 antimicrobial compounds. *Antimicrob. Agents Chemother.* 2006;50(11):3519–3528.

578 12. Grossman NT, Casadevall A. Physiological differences in *Cryptococcus neoformans* strains *in*

579 *vitro* versus *in vivo* and their effects on antifungal susceptibility. *Antimicrob. Agents Chemother.*

580 2017;61(3):1–33.

581 13. Williamson PR, Wakamatsu K, Ito S. Melanin biosynthesis in *Cryptococcus neoformans*. *J.*

582 *Bacteriol.* 1998;180(6):1570–1572.

583 14. Sabiiti W et al. Efficient phagocytosis and laccase activity affect the outcome of HIV-associated

584 cryptococcosis. *J. Clin. Invest.* 2014;124(5):2000–2008.

585 15. Brilhante RSN et al. An alternative method for the analysis of melanin production in

586 *Cryptococcus neoformans sensu lato* and *Cryptococcus gattii sensu lato*. *Mycoses* 2017;60(10):697–

587 702.

588 16. Huang J et al. Activation of antibacterial autophagy by NADPH oxidases. *Proc. Natl. Acad. Sci.*

589 *U. S. A.* 2009;106(15):6226–6231.

590 17. Akoumianaki T et al. *Aspergillus* cell wall melanin blocks LC3-associated phagocytosis to

591 promote pathogenicity. *Cell Host Microbe* 2016;19(1):79–90.

592 18. Kyrmizi I et al. Calcium sequestration by fungal melanin inhibits calcium-calmodulin signalling

593 to prevent LC3-associated phagocytosis. *Nat. Microbiol.* 2018;3(7):791–803.

594 19. Nicola AM et al. Macrophage autophagy in immunity to *Cryptococcus neoformans* and *Candida*

595 *albicans*. *Infect. Immun.* 2012;80(9):3065–3076.

596 20. Panepinto J et al. Sec6-dependent sorting of fungal extracellular exosomes and laccase of  
597 *Cryptococcus neoformans*. *Mol. Microbiol.* 2009;71(5):1165–1176.

598 21. Rodrigues ML, Godinho RMC, Zamith-Miranda D, Nimrichter L. Traveling into outer space:  
599 unanswered questions about fungal extracellular vesicles. *PLoS Pathog.* 2015;11(12):1–6.

600 22. Matos Baltazar L et al. Antibody binding alters the characteristics and contents of extracellular  
601 vesicles released by *Histoplasma capsulatum*. *mSphere* 2016;1(2):1–17.

602 23. Salas SD, Bennett JE, Kwon-Chung KJ, Perfect JR, Williamson PR. Effect of the laccase gene,  
603 CNLAC1, on virulence of *Cryptococcus neoformans*. *J. Exp. Med.* 1996;184(2):377–386.

604 24. Williamson PR. Laccase and melanin in the pathogenesis of *Cryptococcus neoformans*. *Front.*  
605 *Biosci.* 1997;2. doi:10.2741/A231

606 25. Casadevall A, Perfect JR. *Cryptococcus neoformans*. Washington, D.C: ASM Press; 1998:

607 26. Rosas AL et al. Isolation and serological analyses of fungal melanins.. *J. Immunol. Methods*  
608 2000;244(1–2):69–80.

609 27. Pukkila-Worley R et al. Transcriptional network of multiple capsule and melanin genes governed  
610 by the *Cryptococcus neoformans* cyclic AMP cascade. *Eukaryot. Cell* 2005;4(1):190–201.

611 28. Camacho E et al. The structural unit of melanin in the cell wall of the fungal pathogen  
612 *Cryptococcus neoformans*. *J. Biol. Chem.* 2019;294(27):10471–10489.

613 29. Liu L, Tewari RP, Williamson PR. Laccase protects *Cryptococcus neoformans* from antifungal  
614 activity of alveolar macrophages. *Infect. Immun.* 1999;67(11):6034–6039.

615 30. Erb-Downward JR, Noggle RM, Williamson PR, Huffnagle GB. The role of laccase in  
616 prostaglandin production by *Cryptococcus neoformans*. *Mol. Microbiol.* 2008;68(6):1428–1437.

617 31. Yáñez-Mó M et al. Biological properties of extracellular vesicles and their physiological  
618 functions. *J. Extracell. Vesicles* 2015;4(2015):1–60.

619 32. Rodrigues ML et al. Vesicular polysaccharide export in *Cryptococcus neoformans* is a eukaryotic  
620 solution to the problem of fungal trans-cell wall transport. *Eukaryot. Cell* 2007;6(1):48–59.

621 33. Nosanchuk JD, Nimrichter L, Casadevall A, Rodrigues ML. A role for vesicular transport of  
622 macromolecules across cell walls in fungal pathogenesis. *Commun. Integr. Biol.* 2008;1(1):37–39.

623 34. Alanio A, Desnos-Ollivier M, Dromer F. Dynamics of *Cryptococcus neoformans*-macrophage  
624 interactions reveal that fungal background influences outcome during cryptococcal  
625 meningoencephalitis in humans. *MBio* 2011;2(4). doi:10.1128/mBio.00158-11

626 35. Bielska E, May RC. What makes *Cryptococcus gattii* a pathogen?. *FEMS Yeast Res.*  
627 2015;16(1):1–12.

628 36. Hansakon A, Ngamskulrungroj P, Angkasekwinai P. Contribution of laccase expression to  
629 immune response against *Cryptococcus gattii* infection. *Infect. Immun.* [published online ahead of  
630 print: December 23, 2019]; doi:10.1128/IAI.00712-19

631 37. Mukaremera L et al. The mouse inhalation model of *Cryptococcus neoformans* infection  
632 recapitulates strain virulence in humans and shows that closely related strains can possess differential  
633 virulence. *Infect. Immun.* 2019;87(5). doi:10.1128/IAI.00046-19

634 38. Xu L et al. Chemokine and cytokine cascade caused by skewing of the Th1-Th2 balance is  
635 associated with high intracranial pressure in HIV-associated cryptococcal meningitis. *Mediators*

636 *Inflamm.* 2019;2019:1–9.

637 39. Samarasinghe H et al. Genetic factors and genotype-environment interactions contribute to  
638 variation in melanin production in the fungal pathogen *Cryptococcus neoformans*. *Sci. Rep.*  
639 2018;8(1):1–11.

640 40. Lee D et al. Unraveling melanin biosynthesis and signaling networks in *Cryptococcus*  
641 *neoformans*. *MBio* 2019;10(5). doi:10.1128/mBio.02267-19

642 41. Cordero RJB, Camacho E, Casadevall A. Melanization in *Cryptococcus neoformans* requires  
643 complex regulation. *MBio* 2020;11(1). doi:10.1128/mBio.03313-19

644 42. Butler M, Day A. Fungal melanins: a review. *Can J Microbiol. Can. J. Microbiol.* 2011;44:1115–  
645 1136.

646 43. Beardsley J, Sorrell TC, Chen SC-A. Central Nervous System Cryptococcal Infections in Non-  
647 HIV Infected Patients.. *J. fungi (Basel, Switzerland)* 2019;5(3). doi:10.3390/jof5030071

648 44. Ngamskulrungroj P et al. The trehalose synthesis pathway is an integral part of the virulence  
649 composite for *Cryptococcus gattii*. *Infect. Immun.* 2009;77(10):4584–4596.

650 45. Petzold EW et al. Characterization and regulation of the trehalose synthesis pathway and its  
651 importance in the pathogenicity of *Cryptococcus neoformans*. *Infect. Immun.* 2006;74(10):5877–  
652 5887.

653 46. Levine B. Eating oneself and uninvited guests: autophagy-related pathways in cellular defense.  
654 *Cell* 2005;120:159–162.

655 47. Sanjuan MA et al. Toll-like receptor signalling in macrophages links the autophagy pathway to  
656 phagocytosis. *Nature* 2007;450(7173):1253–1257.

657 48. Geng J, Klionsky DJ. The Atg8 and Atg12 ubiquitin-like conjugation systems in  
658 macroautophagy. “Protein Modifications: Beyond the Usual Suspects” Review Series. *EMBO Rep.*  
659 2008;9(9):859–864.

660 49. Trevijano-Contador N, Zaragoza O. Immune response of *Galleria mellonella* against human  
661 fungal pathogens. *J. fungi (Basel, Switzerland)* 2018;5(1). doi:10.3390/jof5010003

662 50. Mylonakis E et al. *Galleria mellonella* as a model system to study *Cryptococcus neoformans*  
663 pathogenesis. *Infect. Immun.* 2005;73(7):3842–3850.

664 51. Firacative C, Duan S, Meyer W. *Galleria mellonella* model identifies highly virulent strains  
665 among all major molecular types of *Cryptococcus gattii*. *PLoS One* 2014;9(8):e105076.

666 52. Bouklas T, Diago-Navarro E, Wang X, Fenster M, Fries BC. Characterization of the virulence of  
667 *Cryptococcus neoformans* strains in an insect model. *Virulence* 2015;6(8):809–813.

668 53. Chen YL, Lehman VN, Lewit Y, Averette AF, Heitman J. Calcineurin governs thermotolerance  
669 and virulence of *Cryptococcus gattii*. *G3 Genes, Genomes, Genet.* 2013;3(3):527–539.

670 54. Garcia-Solache MA, Izquierdo-Garcia D, Smith C, Bergman A, Casadevall A. Fungal virulence  
671 in a lepidopteran model is an emergent property with deterministic features. *MBio* 2013;4(3).  
672 doi:10.1128/mBio.00100-13

673 55. Cunha MM et al. Melanin in *Fonsecaea pedrosoi*: A trap for oxidative radicals. *BMC Microbiol.*  
674 2010;10. doi:10.1186/1471-2180-10-80

675 56. Bach CE et al. Measuring phenol oxidase and peroxidase activities with pyrogallol, l-DOPA, and  
676 ABTS: Effect of assay conditions and soil type. *Soil Biol. Biochem.* 2013;67:183–191.

677 57. Sinsabaugh RL et al. Soil microbial activity in a *Liquidambar* plantation unresponsive to CO 2-

678 driven increases in primary production. *Appl. Soil Ecol.* 2003;24(3):263–271.

679 58. Zaragoza O, Casadevall A. Experimental modulation of capsule size in *Cryptococcus*

680 *neoformans*. *Biol. Proced. Online* 2004;6(1):10–15.

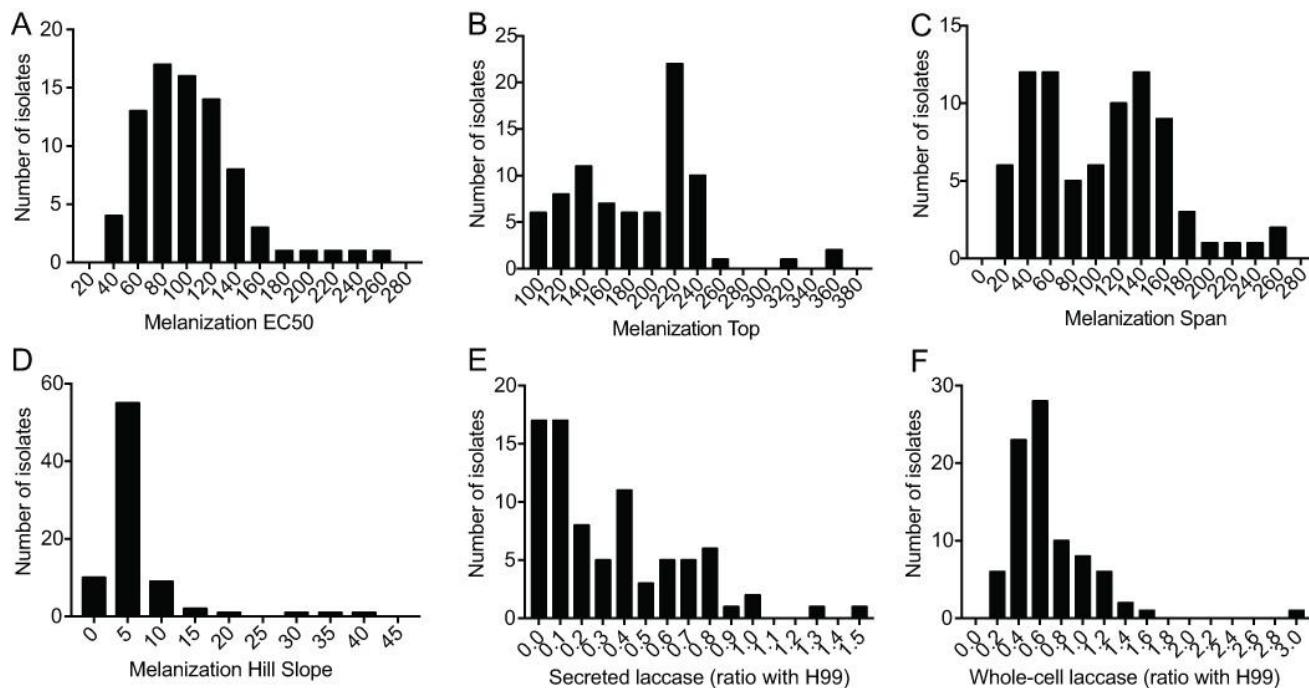
681 59. Ost KS, O'Meara TR, Huda N, Esher SK, Alspaugh JA. The *Cryptococcus neoformans* alkaline

682 response pathway: identification of a novel Rim pathway activator. *PLoS Genet.* 2015;11(4).

683 doi:10.1371/journal.pgen.1005159

684

685 **Figures and figure legends**

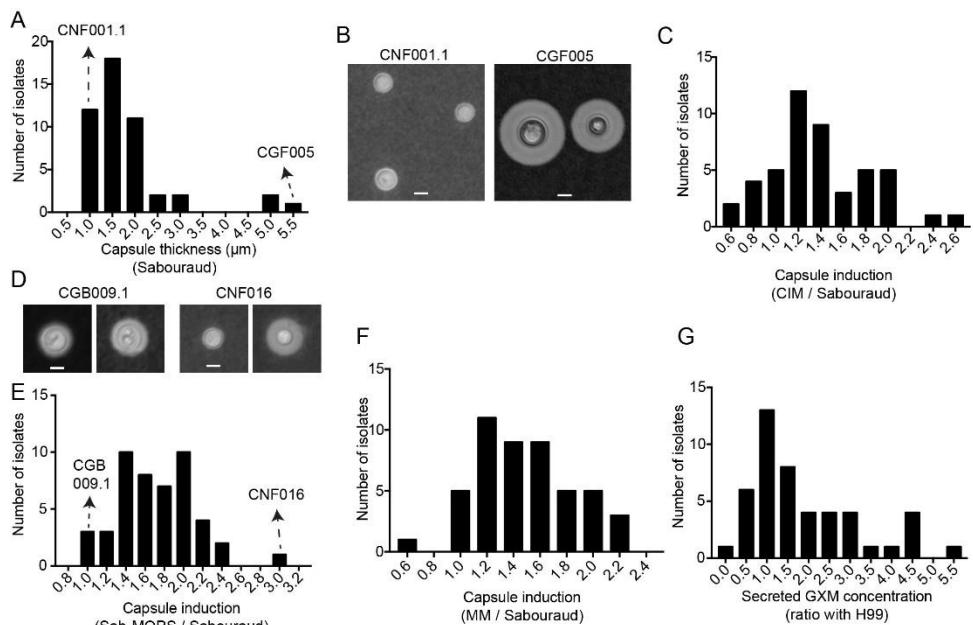


686

687 **Figure 1 – Melanization kinetics of and laccase production by *Cryptococcus* spp. clinical isolates**

688 (A-D) Histograms showing the distribution of the melanization kinetics parameters from clinical  
689 isolates. Images of each colony taken throughout 168 h of incubation in melanizing medium were  
690 processed and fitted to sigmoidal curves to obtain: (A) melanization EC50 - time in hours for the  
691 colony to reach half maximum melanization; (B) melanization Top - median gray level of the colony  
692 at the end of the experiment, which indicates how dark the colony became; (C) melanization Span  
693 (difference in median gray levels of the colony at the beginning and end of the experiment); (D)  
694 melanization Slope (slope of the sigmoidal curve at the inflection point, an expression of how fast the  
695 colony melanizes. (E-F) Histograms showing the specific laccase activity from clinical isolates.  
696 Frequency distribution of secreted laccase activity, n = 82 and (F) Frequency distribution of whole-  
697 cell laccase activity, n = 84.

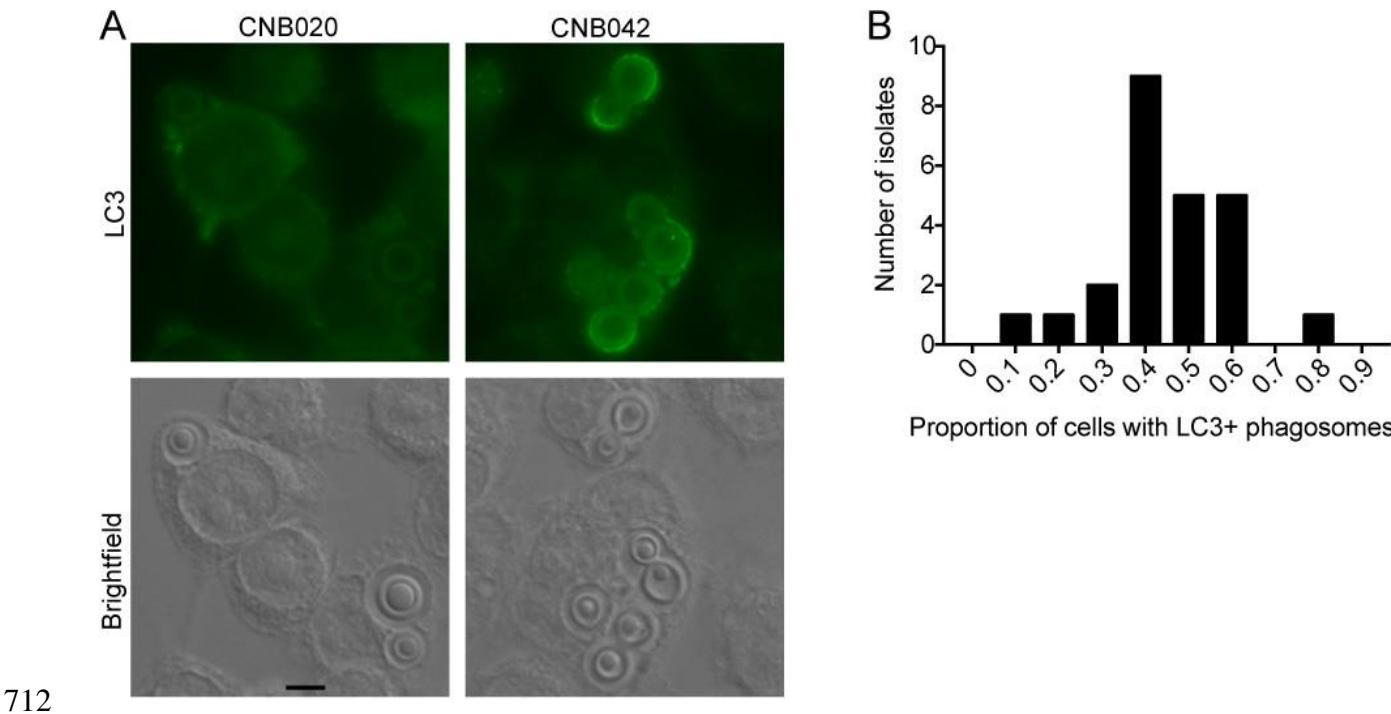
698



699

700 **Figure 2 – Capsule size of and GXM secretion by clinical isolates**

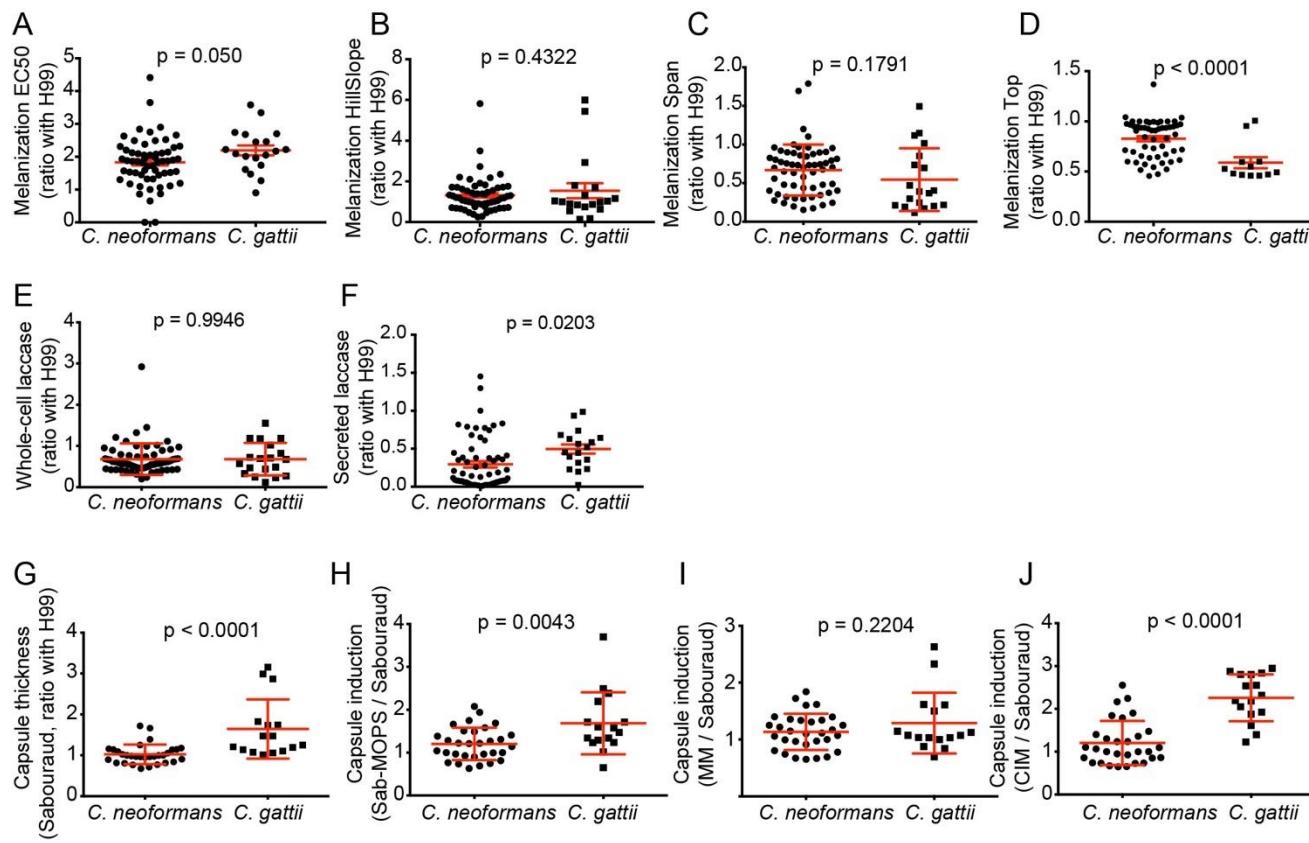
701 (A) Frequency distribution of the capsule thickness in Sabouraud medium (Sab), n = 48. (B)  
702 Representative photo of isolates at both ends of the capsule thickness distribution, CNF001.1 isolate  
703 less than 1 μm thick and CGF005 isolate greater than 5 μm. (C) Frequency distribution of capsule  
704 induction in CO<sub>2</sub>-independent medium (CIM) relative to Sabouraud (CIM / Sabouraud), n = 47. (D)  
705 Representative photo of isolates at both ends of the relative distribution, isolate CGB009.1, which  
706 maintained the same capsule thickness in both media, and isolate CNF016, whose capsule was about  
707 3 times larger in Sab-MOPS media (n = 48) and (E) Frequency distribution of capsule induction in  
708 Sab-MOPS relative to Sabouraud (Sab-MOPS / Sabouraud). (F) Frequency distribution of capsule  
709 induction in Minimum Medium (MM) relative to Sabouraud (MM / Sabouraud), n = 48. (G)  
710 Frequency distribution of secreted GXM concentration, n = 46. Experiments were repeated at least  
711 twice and had similar results. Scale bars: 5 μm.

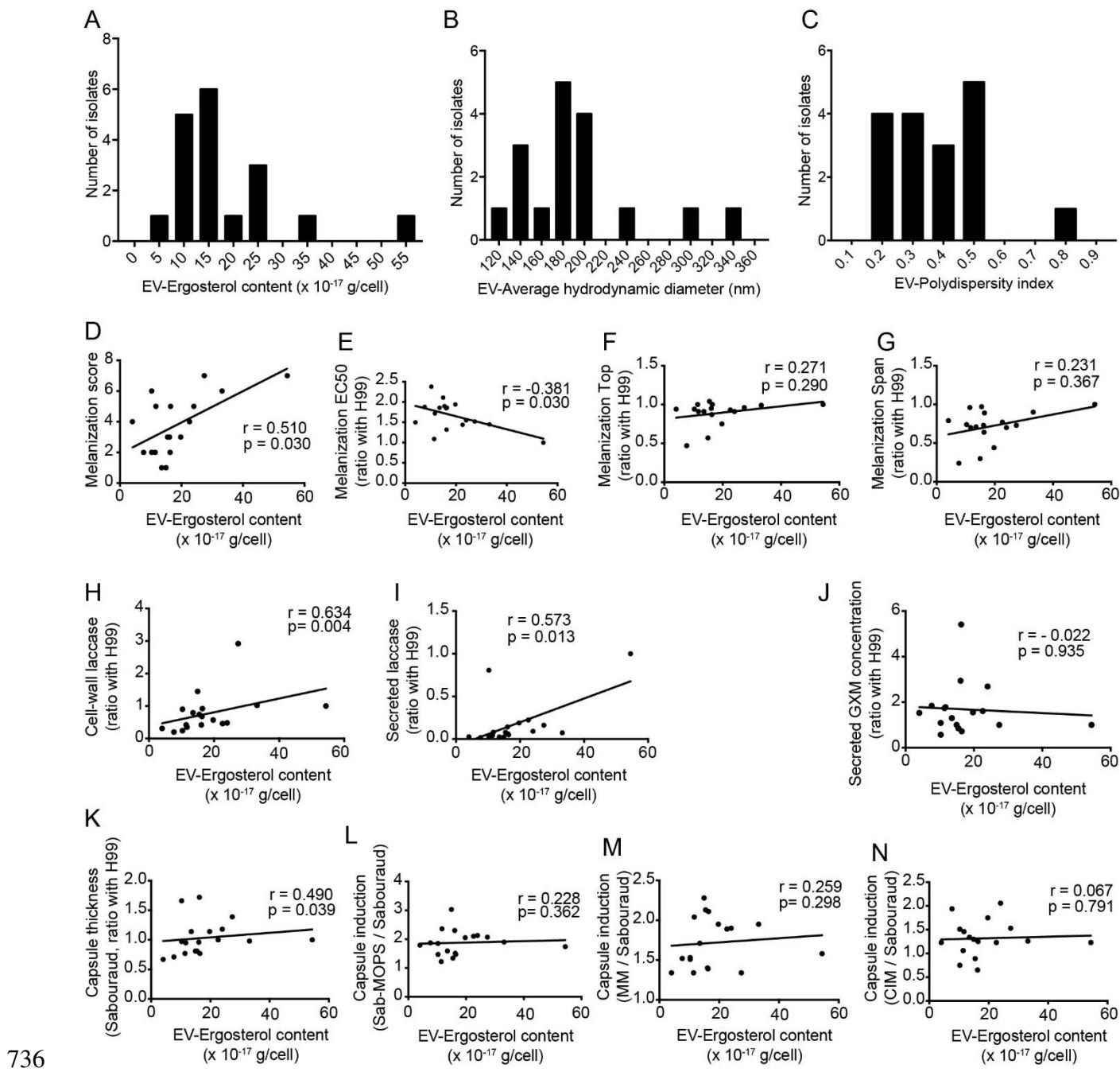


713 **Figure 3 – Interaction of clinical isolates with macrophages in LC3-associated phagocytosis**  
714 **(LAP)**

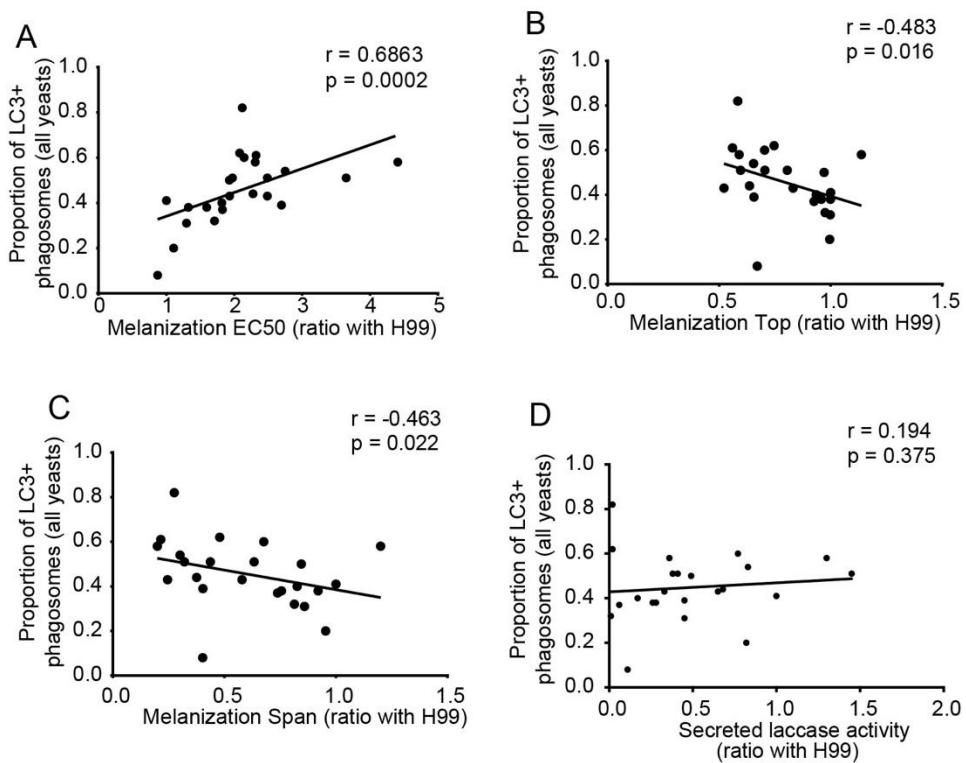
715 Representative photo of the autophagy assessment by immunofluorescence, measured by means of  
716 phagocytosis associated with LC3 (LAP). Clinical isolate CNB020 with low LAP induction and  
717 CNB042 with high LAP induction. (B) Frequency distribution of the proportion of J774 cells with  
718 LC3-positive phagosomes, n =24. Experiments were repeated at least twice in different days and had  
719 similar results. Scale bars: 10  $\mu$ m.

720





744 = -0.381, p = 0.030). (F) No correlation between EV-Ergosterol content with Melanization Top  
745 (maximum melanization index from non-linear regression curve of median gray value) ( $r = 0.271$ , p  
746 = 0.290) or (G) melanization Span (difference in median gray levels of the colony at the beginning  
747 and end of the experiment - difference between top and bottom) ( $r = 0.321$ , p = 0.004). (H)  
748 Correlation between EV-Ergosterol content with cell-wall laccase activity ( $r = 0.634$ , p <0.001) and  
749 (I) secreted laccase activity ( $r = 0.573$ , p = 0.013). (J) No correlation between EV-Ergosterol content  
750 and secreted GXM concentration ( $r = -0.022$ , p = 0.935). (K) Correlation between EV-Ergosterol  
751 content and capsule thickness in medium rich Sabouraud (Sab) ( $r = 0.490$ , p = 0.039). (L) No  
752 correlation between EV-Ergosterol content with capsule induction in medium Sab-MOPS ( $r = 0.228$ ,  
753 p = 0.362), (M) capsule induction in medium minimal ( $r = 0.259$ , p = 0.298) and (N) capsule  
754 induction in CO<sub>2</sub>-independent medium ( $r = 0.067$ , p = 0.791). All samples (n = 18) were analyzed in  
755 duplicate and under the same conditions. All correlations were made with Spearman rank.  
756

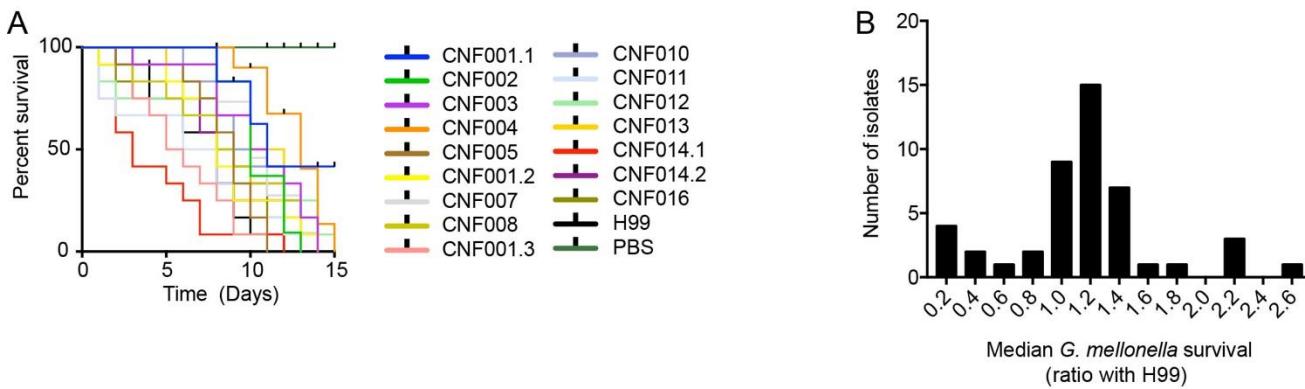


757

758 **Figure 6 – Melanization kinetics affect the ability of clinical isolates to escape from LC3-  
759 associated phagocytosis**

760 Using classical murine macrophages J774, without previous activation stimuli. (A) Correlation  
761 between LC3- associated phagocytosis with melanization EC50 (represents the speed of melanization  
762 index from non-linear regression curve of median gray value) ( $r = 0.686$ ,  $p = 0.0002$ ), (B) with  
763 melanization Top (maximum melanization index from non-linear regression curve of median gray  
764 value) ( $r = -0.483$ ,  $p = 0.016$ ), (C) with melanization Span (difference in median gray levels of the  
765 colony at the beginning and end of the experiment - difference between top and bottom) ( $r = -0.463$ ,  
766  $p = 0.022$ ) and no correlation with secreted laccase activity ( $r = 0.194$ ,  $p = 0.375$ ). All correlations  
767 were made with Spearman rank.

768



769

770 **Figure 7 - *G. mellonella* survival after infection with different *C. neoformans* isolates**

771 (A) Survival curve of *G. mellonella* infected with clinical isolates. In this experimental batch, all  
772 isolates were *C. neoformans* of the molecular type VNI. (B) Frequency distribution of the median  
773 survival *G. mellonella* infected with clinical isolates (n = 46). Twelve individuals were infected per  
774 isolate.

775

776

777 **Tables**

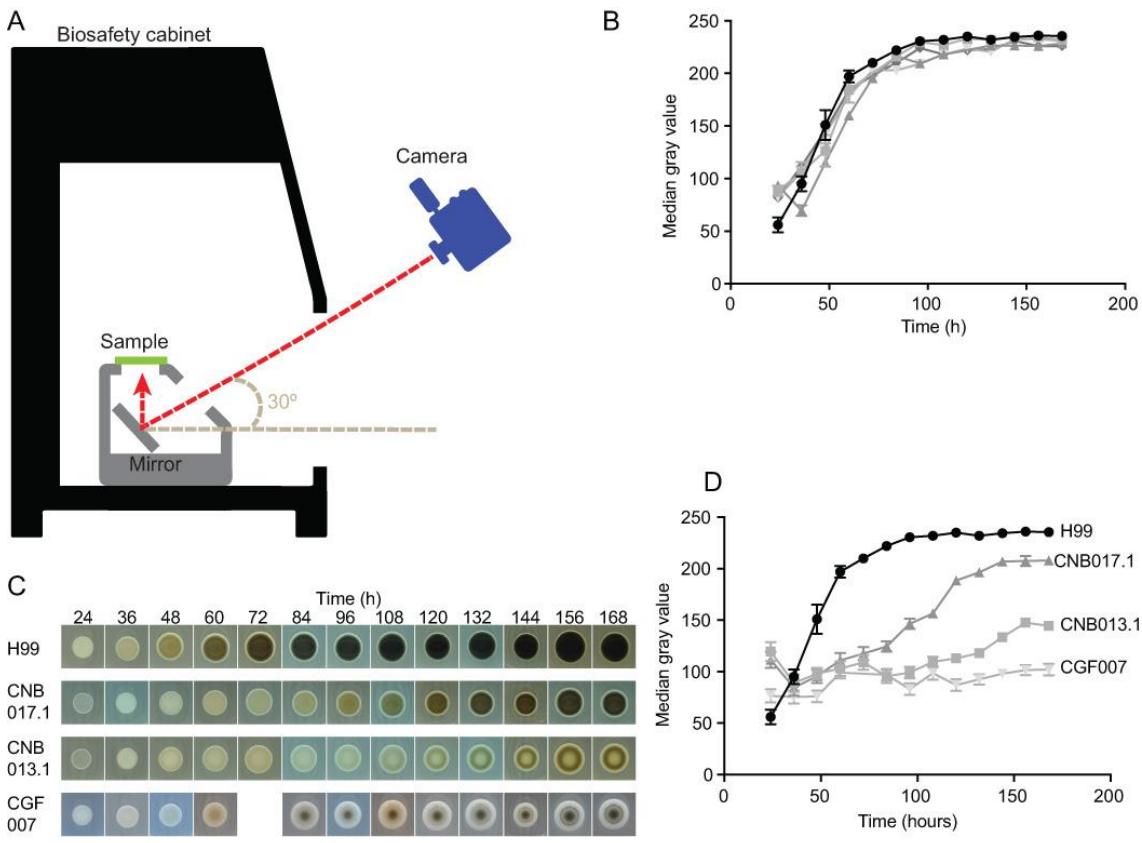
778 **Table 1 – Patient characteristics**

<b>Age (years)</b>	$42 \pm 17.7$ (mean $\pm$ Std. Dev.)
<b>Gender</b>	68.3% male, 31.7% female
<b>HIV infection status<sup>A</sup></b>	68.3% positive, 29.3% negative, 2.4% unknown
<b>CD4 count (cells per mm<sup>3</sup>)<sup>B</sup></b>	$71 \pm 78.9$ (median $\pm$ Std. Dev.)
<b>Other risk factors<sup>C</sup></b>	9.8%
<b>Apparently immunocompetent<sup>D</sup></b>	19.5%
<b>Intracranial hypertension<sup>E</sup></b>	48.6% - Yes; 10.8% - No; 40.6% - no information
<b>Poor prognosis criteria<sup>F</sup></b>	58.5%
<b>Two week mortality<sup>G</sup></b>	30.7%
<b>Ten week mortality<sup>H</sup></b>	41%

779 <sup>A</sup>HIV infection determined by serological testing. <sup>B</sup>CD4 cell counts in the peripheral blood of 18 out of 28 HIV-positive  
780 patients. <sup>C</sup>Proportion of the 41 patients which had at least one of the following risk factors: diabetes, use of  
781 corticosteroids, use of other immunosuppressive drugs or primary immunodeficiencies. <sup>D</sup>Proportion of the 41 patients that  
782 were HIV-negative and had no other known immunosuppression. <sup>E</sup>Proportion of the 37 patients with CNS disease that  
783 had intracranial hypertension upon the diagnosis of the disease, defined as CSF opening pressure of more than 25 mmHg  
784 or papilledema on ophthalmoscopy. <sup>F</sup>Proportion of the patients that had at least one of the following signs or symptoms:  
785 confusion, lowered consciousness, coma or focal neurological deficits. <sup>G</sup>Proportion of the 39 patients that were followed  
786 for at least two weeks that died before or on the 14<sup>th</sup> day after the diagnosis. <sup>H</sup>Proportion of the 39 patients that were  
787 followed for at least ten weeks that died before or on the 70<sup>th</sup> day after the diagnosis.

788

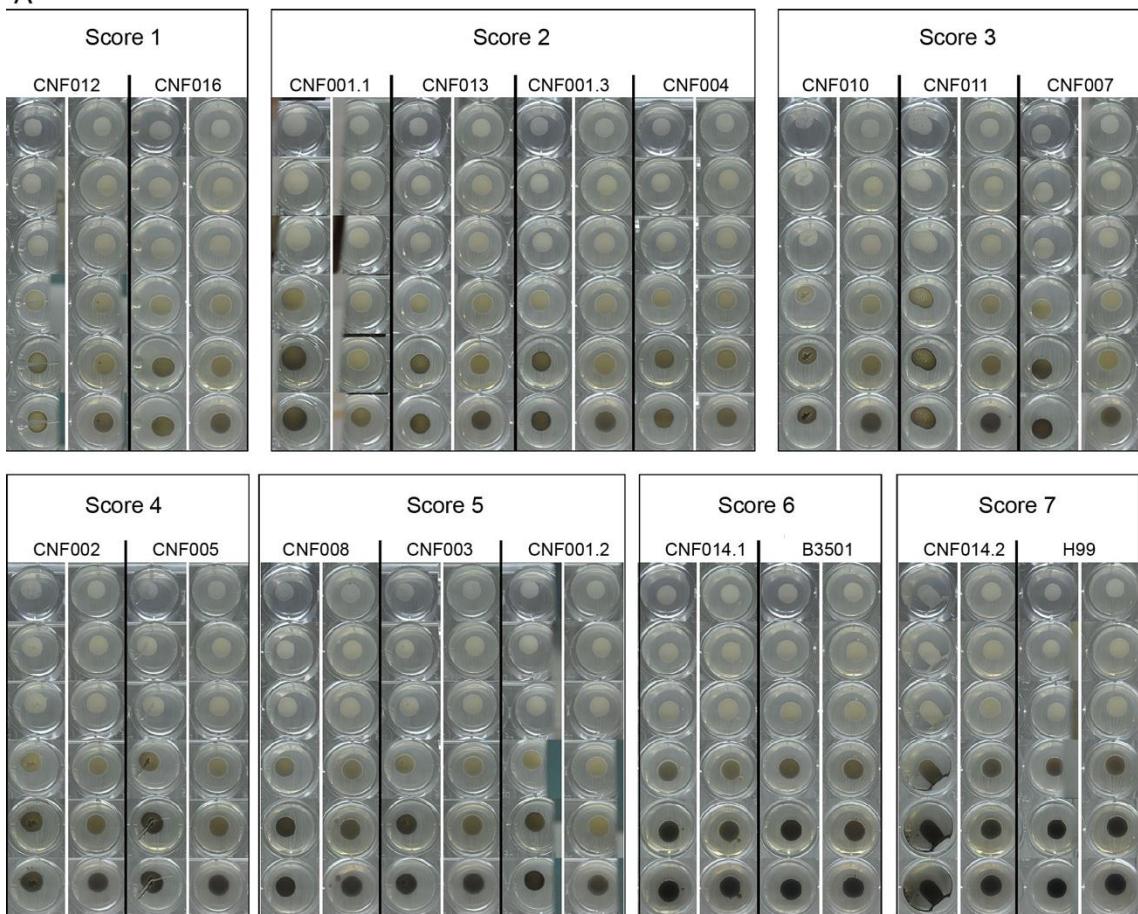
789 **Supplemental data**



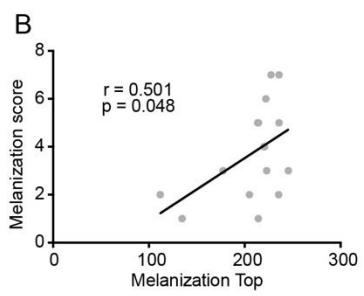
791 **Figure S1**

792 Figure legend. Reproducibility of the new method for quantifying melanin and colony melanization  
793 profile. (A) Colony image capture system (mirror plate reflector device developed and manufactured  
794 in our research group). (B) Five biological replicates of H99 performed in duplicate technique reveal  
795 high reproducibility of the methodology developed to quantify melanin. Three image processing  
796 programs were used (Adobe Photoshop CC version 19.0, ImageJ version 1.50i and Prism 7 version  
797 7.0a, respectively) to calculate the median gray values of each colony over time. (C) Representative  
798 photo of the macroscopic melanization profile of three clinical isolates and the standard H99 internal  
799 control strain in all experiments. Two strains with homogeneous colony melanization (H99 and  
800 CNB017.1) and two heterogeneous (CNB013.1 and CGF007). (B and D) Non-linear regression curve  
801 of median gray value (represents amount of melanin measured at each observed time).  
802

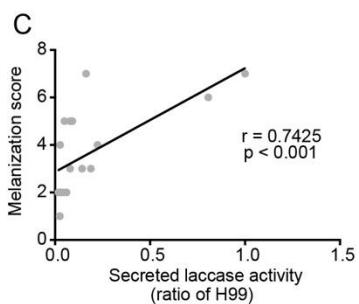
A



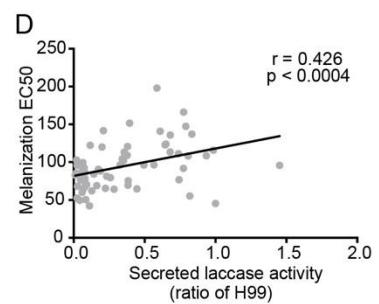
B



C



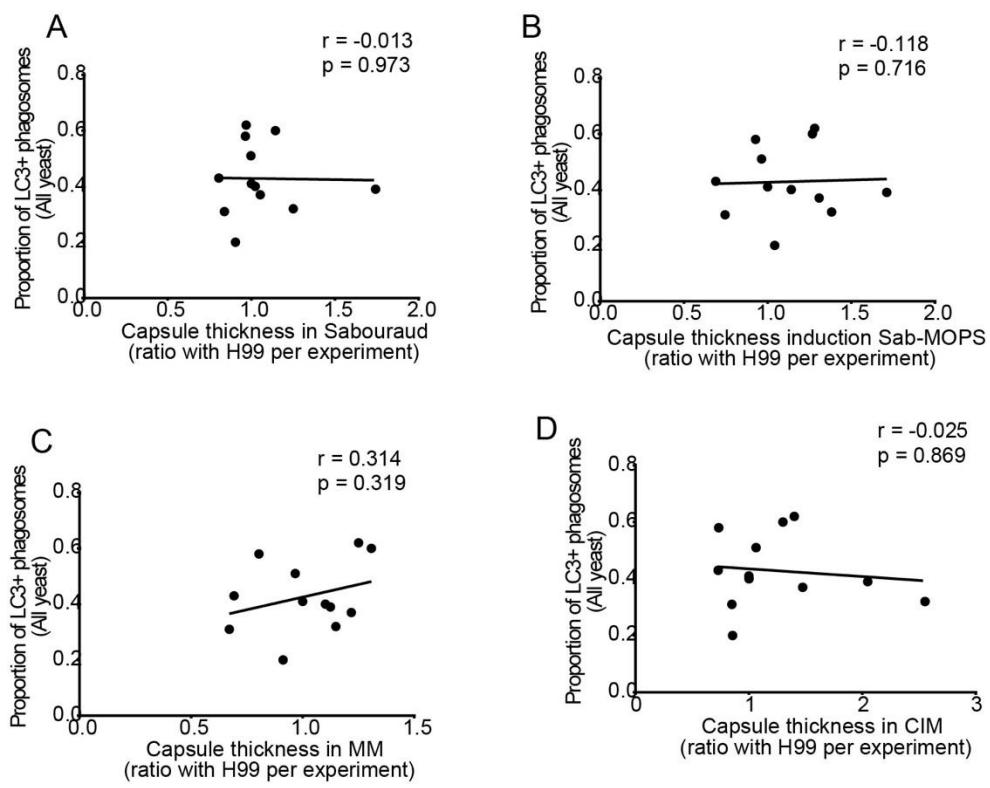
D



803

804 **Figure S2**

805 Figure legend. Validation and correlation of the new method for quantifying melanin. (A) Classic  
 806 semi-quantitative melanization score. The isolates were qualitatively categorized into 7 groups  
 807 ordered from 1 to 7. Group 1 contains the colony with smaller and later melanization and group 7 the  
 808 most effective isolates for melanin production. (B) Correlation between Melanization Top (maximum  
 809 melanization index from non-linear regression curve of median gray value) and classical  
 810 melanization score ( $r = 0.501, p = 0.048$ ). (C) Correlation between secreted laccase activity and  
 811 classical melanization score ( $r = 0.74, p < 0.001$ ). (D) Correlation between secreted laccase activity  
 812 and Melanization EC50 (represents the speed of melanization index from non-linear regression curve  
 813 of median gray value) ( $r = 0.426, p < 0.0004$ ). Melanization score defined with double blind control  
 814 of two experienced individuals. All correlations were made with Spearman rank.  
 815



818 Capsule thickness no affect the ability of clinical isolates to escape from LC3-associated  
819 phagocytosis. (A) No correlation between LC3-associated phagocytosis with capsule thickness in  
820 medium rich Sabouraud (Sab) ( $r = -0.013$ ,  $p = 0.973$ ), (B) with capsule induction in medium Sab-  
821 MOPS ( $r = -0.118$ ,  $p = 0.716$ ), (C) capsule induction in medium minimal ( $r = 0.314$ ,  $p = 0.319$ ) and  
822 (D) capsule induction in medium CO<sub>2</sub> independent ( $r = -0.025$ ,  $p = 0.869$ ). All correlations were  
823 made with Spearman rank.