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**Dormancy in *Cryptococcus neoformans*: sixty years of accumulating evidence**

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**Abstract (125 words)**

*Cryptococcus neoformans* is an opportunistic yeast that is present worldwide and interacts with various organisms. In humans, it is responsible for cryptococcosis, a deadly invasive fungal infection which represents around 220,000 cases per year worldwide. Starting from the natural history of the disease in humans, there is accumulating evidence on the capacity of this organism to enter dormancy. In response to the harsh host environment, the yeast is able to adapt dramatically and escape the vigilance of the host's immune cell to survive. Indeed, the yeast exposed to the host takes on pleiotropic phenotypes, enabling the generation of populations in heterogenous states, including dormancy, to eventually survive at low metabolic cost and revive in favorable conditions. The concept of dormancy has been validated in *C. neoformans* from both epidemiological and genotyping data, and more recently from the biological point of view with the characterization of dormancy through the description of viable but non-culturable cells.

39 *Cryptococcus neoformans* is basidiomycetous opportunistic yeast that is widely present  
40 in the environment. It causes human cryptococcosis, which mainly affects  
41 immunocompromised patients and presents as a meningoencephalitis (1) that is lethal  
42 without treatment. Clinical presentation is often diagnosed late because clinical  
43 symptoms are initially mild with a sub-acute to chronic evolution (2).

44 Humans are exposed to *C. neoformans* from the environment. In nature, this fungus can  
45 survive the predation of various organisms ranging from protozoans to metazoans  
46 through ready-made virulence traits (3). *C. neoformans* interacts closely with uni- or  
47 multi-cellular organisms (2–4) and with cells dedicated to innate immune responses in  
48 metazoans (macrophages, dendritic cells, natural killer lymphocytes) with various  
49 propensity to be phagocytosed and killed (4–6). *C. neoformans* is a facultative  
50 intracellular pathogen (7). Interaction of *C. neoformans* with host cells can lead to  
51 phagocytosis, yeast replication within the phagolysosome, and is sometimes associated  
52 with host cell lysis or with non-lytic exocytosis or cell-to-cell transfer and eventually killing  
53 of the yeast (8–13). These phases have been well studied in different models of interaction  
54 with host cells but mainly within macrophages. Indeed, intracellular persistence and  
55 multiplication in immune cells provide advantages to the fungus by allowing escape from  
56 the immune response and later dissemination through epithelial barriers (14, 15).

57 Characteristics of the infection depend on both hosts and microbial factors. Fungal  
58 factors described as virulence factors influence the outcome of infection, according to  
59 data obtained in the mouse model of cryptococcosis (16), but also in vitro (17) and in  
60 humans (8, 18). Microbial adaptation to the hosts is complex and has been studied  
61 globally in lungs using histopathology (7) and global transcriptome analysis upon amoeba  
62 (19) and macrophage ingestion (20) or upon early infection of mice and rabbits (21, 22).

63 Quiescence or dormancy is one of such adaptation that appears successful for  
64 enhancing the fungus's ability to survive, persist, reactivate, and then disseminate (23).

65 About sixty years of research focusing on how *C. neoformans* is able to cause infection  
66 in humans is available in literature, leading to the recent biological demonstration of  
67 dormancy in this organism. This review aims to summarize these sixty years of research,  
68 starting from the knowledge of human infection and ending with the characterization of

69 dormancy biologically. This review is assembled to make the reader understand how this  
70 knowledge has been integrated to lead to more recent findings on the biology of *C.*  
71 *neoformans* characterizing dormancy, focusing on (i) The description of the natural  
72 history of *C. neoformans* infection in humans; (ii) The concept of dormancy in Fungi; (iii)  
73 Dormancy in *C. neoformans* in vivo and (iv) in vivo. To finish, a section is dedicated to  
74 discuss the (vi) relevance of the biological findings regarding human infection and to bring  
75 unsolved questions that can be the bases of future work in the field.

## 76 **1. Natural history of cryptococcosis in humans**

77  
78 Cryptococcosis is one of the most frequent fungal invasive infection in humans  
79 worldwide (24). The vast majority of patients with cryptococcosis are HIV-positive  
80 patients, mostly those with CD4 T cells <100 cells per  $\mu\text{L}$ . Nevertheless, in western  
81 countries, the number of cryptococcosis cases recorded in HIV-negative individuals  
82 becomes higher than that in HIV-positive patients (25). Immunocompromised HIV-  
83 negative patients at risk of cryptococcosis are mainly solid organ transplant recipients,  
84 patients with systemic autoimmune disease, and those with hematological malignancies  
85 (26).

86 The natural history of cryptococcosis is described following two main routes. The first  
87 one, although rare, occurs after exposure to *C. neoformans* while immunocompromised,  
88 leading to rapidly progressive cryptococcosis; the second one is reminiscent of  
89 tuberculosis, with a phase of latency with reactivation and dissemination. This second  
90 route appears to be the main mechanism of infection and so will be further developed in  
91 this review.

### 92 93 1.a. First route of infection: ready-made for disease

94  
95 Confronted with the need to survive in nature and to survive different hosts in different  
96 environments, *C. neoformans* has selected ready-made virulence traits (3). From a  
97 deterministic point of view, *C. neoformans* population also needs diversity to survive

98 predators harboring different killing propensities. The plasticity of the *C. neoformans*  
99 genome could lead to this diversity (27). *C. neoformans* and *C. gattii* are haploid  
100 organisms that can be found as diploid organisms both in nature and in hosts (28, 29).  
101 Generation of hybrids is possible between the varieties *grubii* (serotype A) and  
102 *neoformans* (serotype D) (29, 30) but also between *C. neoformans* and *C. gattii*, again  
103 illustrating this plasticity (31).

104 *C. neoformans* has long been associated with pigeon droppings (32). Indeed, pigeon  
105 fanciers are known to have higher anti-*C. neoformans* antibodies than control individuals  
106 (33). The presence of *C. neoformans* in human dwellings was a risk factor (odds ratio =  
107 2.05) for the development of cryptococcosis in HIV-positive patients from Brazil (34). *C.*  
108 *gattii* has also been found in indoor environments in Brazil, although links with human  
109 cryptococcosis have not been demonstrated (35). Several cases of cryptococcosis have  
110 been reported in immunosuppressed patients in contact with birds (pigeons, parrot,  
111 cockatoo, cockatiel) (36–39). The presence of *C. neoformans* in the feces of some animals  
112 have been also observed in zoo animals (*Guizotia abyssinica*, Palm Cockatoo, Military  
113 Macaw, Gray Parrot) (40). Nosocomial cases of cryptococcosis acquired in various  
114 hospital settings have also been suspected (41, 42). Transmission of *C. neoformans*  
115 through transplanted deep organs from a contaminated donor has occurred (43, 44) with,  
116 in some cases, the demonstration of the same strain in different patients transplanted  
117 with organs from the same donor (45).

118 Primary cryptococcosis initiates with lung involvement and then disseminates from the  
119 lung in immunocompromised hosts. Primary pulmonary cryptococcosis is observed in  
120 immunocompetent and immunocompromised hosts. It can be recognized within a broad  
121 range of presentation, from isolated asymptomatic nodules that can mimic cancer lesions  
122 to more disseminated lesions of the lung with respiratory failure (46–48). Primary  
123 cutaneous cryptococcosis is also a clinical entity that happens after environmental  
124 inoculation in immunocompetent or immunocompromised hosts (49, 50).

125

126

127 1.b. Second route of infection: ready-made for latency

128

129 The majority of the infections arise from a natural history of the infection following 3 steps:  
130 primary infection in childhood and immune control, followed by a silent phase of latency  
131 that can last for years, and finally, reactivation and dissemination that are responsible for  
132 the symptoms of the disease mainly occurring upon immunosuppression.

133

#### 134 Early environmental exposure

135 Inhalation of aerosolized particles from soil (desiccated yeasts or basidiospores) is  
136 thought to be the major route of infection in humans (51). Primary infection with *C.*  
137 *neoformans* occurs mainly in immunocompetent children as demonstrated by serological  
138 studies with unrecognized (asymptomatic) infection as the main clinical presentation. The  
139 proportion of children immunized against *C. neoformans* increases with age. Acquisition  
140 of cryptococcal antibodies begins very early (1 year) with minimal reactivity of the sera.  
141 After 5 years, 70% of children react with *C. neoformans* antigens (52). However, the  
142 acquisition of anti-cryptococcal humoral immunity varies among geographic areas.  
143 Cryptococcal antibodies are very common in Bronx children but not in another New York  
144 areas (Dutchess County), nor are they common in Manila (The Philippines), another  
145 densely populated urban area (53). Environmental exposure may depend on climatic and  
146 environmental factors (temperature, humidity, pigeon density), but also on human  
147 sociological factors (habitat conditions, financial resources). These findings support  
148 epidemiological data revealing that cryptococcosis in immunocompromised individuals  
149 is more prevalent in some areas of the world, especially in Africa (24, 54).

150 Of note, *C. gattii* exposure and primoinfection does not follow the same epidemiological  
151 trends than *C. neoformans* based on studies realized in endemic areas in animals and  
152 humans (55, 56).

153

#### 154 Latency

155 Serologic evidence of early cryptococcal immunity in immunocompetent hosts without  
156 recognized infection seems paradoxical considering the very low frequency of

157 cryptococcosis in immunocompetent hosts. However, immune control of the yeasts by  
158 immunocompetent hosts following primoinfection is possible, with latency of the disease  
159 or complete clearance of the fungus as a consequence. Immunocompetent adults  
160 frequently exposed to *C. neoformans* had positive skin test but did not develop clinical  
161 disease (57). Autopsy studies have raised the hypothesis that pulmonary granulomas  
162 could be the site for persistence because *C. neoformans* is observed in sub-pleural  
163 nodules and draining lymph nodes in immunocompetent and immunocompromised  
164 hosts (58). Indeed, several reports showing that *C. neoformans* lymphadenitis have been  
165 exclusively found and isolated from lymph node can be found in literature, thus arguing  
166 that initial immune control of the yeasts operates in lymph nodes (59–65). From recent  
167 and old reports, the lymph nodes associated with lymphadenitis correspond to  
168 granuloma composed of epitheloid cells, Giant cells, and necrosis surrounded by a T cell  
169 infiltrate together with yeasts (64, 66, 67).

170 Analysis of clinical isolates of *C. neoformans* var. *grubii* recovered in France from patients  
171 born in Africa (who moved to France with a median of 110 months elapsing before  
172 isolation of the yeast in France), revealed that yeast genotypes from these patients  
173 clustered together, distinct from the yeast genotypes recovered from patients born in  
174 Europe (68). This study is the main epidemiological evidence for this latency stage of the  
175 disease. This latency can be translated into the capacity of dormancy of the yeasts, which  
176 appears to be the more plausible explanation from the point of view of the biology of the  
177 organism. The same conclusion is also drawn from a serologic survey of solid organs  
178 transplant recipients (immunocompromised hosts). Interestingly, sera obtained before  
179 and after transplantation from transplanted patients with cryptococcosis was compared  
180 to control transplanted patients without history of cryptococcosis. Among patients with  
181 cryptococcosis, half exhibited antibody reactivity against *C. neoformans* only after  
182 transplantation. This suggests that this half of the patients were exposed and developed  
183 the disease after transplantation during immunosuppression. But for the other half of the  
184 patients, antibody reactivity against *C. neoformans* was found before transplantation;  
185 these patients' early development of cryptococcosis after transplantation suggests that  
186 reactivation and dissemination occur rapidly after transplantation from a preexisting



187 isolate in transplant recipient, thus validating again the latency phenomenon (69).  
188 Additionally, report of *C. gattii* infections in patients who travelled to endemic areas years  
189 or months prior to the Vancouver Island *C. gattii* outbreak provides more evidence for  
190 latency (70).

191  
192 Reactivation

193 The first manifestation of reactivation is observed in HIV-infected individuals in whom  
194 asymptomatic cryptococcal antigenemia is detected (71–73). Viable yeasts are not  
195 recovered from clinical sample at this step but treatment is mandatory to prevent  
196 symptoms and dissemination (74, 75). Pulmonary cryptococcosis is a well-described  
197 clinical entity that can evolve differentially depending on the immune status of the hosts.  
198 In immunocompetent hosts, *C. neoformans* does not usually disseminate, whereas the  
199 possibility of dissemination in immunocompromised patients is high. It is likely that  
200 dissemination occurs after reactivation of lung-persistent yeasts, crossing the lung  
201 epithelial barrier and disseminating through capillary blood (76, 77). However, abnormal  
202 chest X-ray or CT scan was observed in 39% of HIV-positive patients and 55% of HIV-  
203 negative patients at diagnosis, although dissemination represented 60.6% and 38.5% of  
204 the cases, respectively (1). However, pulmonary symptoms are not the main clinical  
205 manifestation of cryptococcosis in immunocompromised patients. Indeed, most are  
206 diagnosed at the stage of dissemination or meningoencephalitis (1). Cryptococcosis is  
207 characterized by a high frequency of central nervous system (CNS) involvement with  
208 positive cerebrospinal fluid (CSF) and dissemination through blood. Cryptococcosis is  
209 more severe in male HIV-positive patients and those infected with *C. neoformans*  
210 serotype A (1). Acute cryptococcal meningoencephalitis (CM) is always fatal without  
211 antifungal therapy (78). Treatment of CM requires an antifungal therapy induction based  
212 on amphotericin B and flucytosine (79). Based on recent large clinical trials in African  
213 settings, one-week amphotericin B combined with oral flucytosine followed by high-dose  
214 fluconazole is now recognized as the reference therapy (75, 80). Mycological failure after  
215 2 weeks of induction is recognized as a factor of bad prognosis, which requires  
216 continuation of the induction therapy (79). Mycological failure is independently

217 associated with initial dissemination, high serum antigenemia (>1:512), and lack of initial  
218 flucytosine treatment (1, 80, 81). The three-month mortality rate during the management  
219 of acute cryptococcal meningoencephalitis approximates 15-20% in western countries  
220 despite adequate treatment and management. It is still not clear whether this mortality  
221 rate is due to individual's immune status, genetic factors (82), fungal determinants, or a  
222 combination of these. Nevertheless, two reports clearly identified that fungal  
223 determinants specific to the strain are responsible for a given phenotype of interaction  
224 with host cells (high phagocytosis, high intracellular proliferation) that is associated with  
225 mortality in patients (8, 83)

226

## 227 **2. The concept of dormancy in Fungi**

228

229 All microorganisms are exposed to periodic constraint conditions and react by inhibiting  
230 their growth, entering into a non-replicative state called quiescence or dormancy (84, 85).  
231 Three main strategies can be delineated in these conditions. The first is the "bust and  
232 bloom" strategy (85), where the microorganism population will grow rapidly with growth  
233 maximization, but upon nutrient exhaustion, the majority of the individuals will die, with  
234 only few cells surviving. These residual cells will resume growth rapidly upon exposure  
235 to nutrient (86). The second strategy is "quiescence," where the bulk of the population  
236 exposed to nutrient-limited environment will arrest or slow growth to enter a viable, non-  
237 replicating state for a long time. This can last month or years for *Mycobacterium*  
238 *tuberculosis* (87). These cells keep a baseline and specific metabolic capacity, maintain  
239 their membrane potential, and do not undergo major morphological change (88). The  
240 third strategy is called "true dormancy," with sporulation as the purest form, in which an  
241 asymmetrical replication leads to the formation of a metabolically inactive spore (89). The  
242 spore harbors specific morphology but shares some biological features with quiescent  
243 cells.

244 Quiescence in *S. cerevisiae* has been studied for a long time. Recently, a strain of *S.*  
245 *cerevisiae* has been found "alive" in bottles of beer and Champagne from the 18th  
246 century found in a shipwreck in the Baltic sea suggesting this phenomenon can last for

247 years in specific conditions. Quiescent yeasts are mainly obtained from cultures grown  
248 to saturation in glucose-rich media (stationary phase) where all nutrients have been  
249 consumed. Different phases have been described, including (i) a first phase of glycogen  
250 production upon rarefaction of glucose (at about 50%) (90), followed by (ii) the regulation  
251 of trehalose before and after glucose exhaustion. Then, (iii) the yeasts undergo a phase  
252 of diauxic shift (following glucose depletion) where growth is slow and metabolism is  
253 adapted to limitation of nutrients, relying on respiratory growth of non-fermentable  
254 sugars such as ethanol or acetate with switch towards respiration, fatty acid pathway,  
255 and glyoxylate cycle pathway and, as a consequence, increased formation of antioxidant  
256 defenses (scavenging of ROS) (91). The yeast population obtained in stationary phase is  
257 described as a heterogenous population including quiescent cells (composed of  
258 daughter and young mother cells) but also non-quiescent cells, which lose their ability to  
259 accumulate ROS, exhibit genomic instability, and become senescent or apoptotic (92)  
260 In *C. neoformans*, growth arrest in G1 or G2 period has been demonstrated in stationary  
261 phase (93). No specific morphological differences in the mitochondrial apparatus was  
262 observed in logarithmic versus stationary phase (Figure 1) (94). No comprehensive  
263 analysis on the metabolism of *C. neoformans* in stationary phase compared to  
264 logarithmic phase existed until recently, as part of the investigation of a specific  
265 phenotype observed upon exposure to drastic conditions (95).

266

### 267 **3. Dormancy in *C. neoformans* in vivo**

268

269 The body of evidence for dormancy elaborates on various parameters, mainly including  
270 viability, which should not be based on culturability, reactivation upon specific stimuli,  
271 and specific biological activity. Viability requires the use and adaptation of tools available  
272 to test viability/death in mammalian cells (23). For a long time, viability and its corollary  
273 (killing or death of *C. neoformans*) was investigated using CFU counting (96). Other  
274 means to assess the viability/death of yeast have now been developed, including the use  
275 of intercalating dyes such as propidium iodide that is able to diffuse and stain the DNA  
276 of the yeasts only if the extracellular membrane lose integrity (97) This method allows

277 assessment of viability or death by using flow cytometry. Other intercalating dyes can be  
278 used with the same principle (23, 98). These methods assume that a dead yeast cell will  
279 lose membrane integrity, which is potentially not necessary at first. Apoptosis should also  
280 be checked in the context to determine if the cell is oriented towards cell-death or will  
281 remain viable. The existence of apoptosis in fungi is debated (99), but evidence exists for  
282 the presence of caspase-like proteins in *C. neoformans* (100) that could act as effectors  
283 of mechanisms related to caspase-dependent cell death. Nevertheless, apoptosis in  
284 Fungi cannot be directly equated to what is known in mammalian cells (99, 101).

285 In *C. neoformans*' stationary phase, it was shown that only a small proportion of the  
286 population was unable to grow and considered dead (23). From *in vivo* experiments and  
287 interaction with macrophages, it has been shown that yeast cells were able to keep their  
288 round shape and capsule, although dead as shown with different means (23). These dead  
289 *C. neoformans* yeast cells have been called DropCn due to the presence of a large central  
290 vesicle inside the cell. The cell wall was shown to be thicker than stationary phase yeasts  
291 (Figure 1). In those dead cells, the intracellular content is collapsed around vesicles  
292 including remaining membranes (stained with MDY64), nucleic acids (stained with  
293 SYTO85), but with no organized nucleus (negative DAPI staining) and no mitochondria  
294 (negative Mitotracker staining) (23). These cells were able to retain a CMFDA staining  
295 (glutathione staining) in their remaining capsule and cell wall, which was supposed to be  
296 intracellular, producing fluorescence artefacts, allowing detection despite being dead. To  
297 prevent such bias, multispectral imaging flow cytometry was used, allowing observation  
298 of the fluorescence within the cells to assess location (23). Apart from those dead cells,  
299 this study highlighted that heterogeneous population of yeasts were generated in the lung  
300 of infected mice and upon macrophage interaction. Indeed, the view of the existence of  
301 homogenous population of yeasts in specific conditions turns out to be inadequate and  
302 raise the question of the accuracy of studies dealing with global analysis of the global  
303 population of yeasts recovered in specific settings. Nevertheless, global transcription  
304 analyses supported the idea of fungal adaptation to hostile environments such as the  
305 macrophage phagolysosome (19, 20, 102), inside amoeba (19), in the lung during murine

306 infection (21), in the central nervous system of rabbit (22), or in human cerebrospinal fluid  
307 (103).

308 Heterogenous populations generated during murine infection included (i) active yeasts  
309 able to bud and multiply, (ii) dead yeasts, and also (iii) a population of more dormant  
310 yeasts. These dormant yeasts were less prone to grow as compared to stationary phase,  
311 which is already considered as a state where almost of the yeasts are quiescent. This  
312 explain why these cells have been called dormant instead of quiescent cells (23). These  
313 cells had also a decreased response to stress (low glutathione production), increased  
314 mitochondrial expression, increased autophagy, and decreased gluconeogenesis-  
315 associated transcriptional activity (23).

#### 316 **4. Dormancy in *C. neoformans* studied in vitro**

317  
318 In the previous study using the mouse model, as few as  $10^4$  dormant yeast cells were  
319 able to be generated after pooling several mice lungs, which is obviously insufficient to  
320 study basic biological processes allowing the characterization of dormancy. Therefore,  
321 the authors worked on an in vitro model to be able to generate a high number of dormant  
322 yeast cells. Recently, the authors released and studied the standardized conditions  
323 allowing the generation of yeast cells harboring a phenotype close to that dormant cells  
324 generated in the lungs of infected mice (95). These conditions are based on a  
325 combination of conditions (low oxygen and limited nutrients) inspired from the Wayne  
326 and the Loebel models (two well-documented conditions enabling the generation of  
327 quiescent *M. tuberculosis*) (104). After stationary phase in YPD and exposure to  
328 anaerobiosis and nutrient starvation during 8 days, the authors observed that 95% of  
329 yeast cells were viable, with few dead cells. They demonstrated that cells were not  
330 apoptotic upon TUNEL staining. Overtime, these yeast cells showed a decreased  
331 culturability on YPD agar plates, ending with about 1% of the cells still able to grow on  
332 agar at Day 8 of incubation. The phenotype observed in the in vivo subpopulation was  
333 resumed with delayed growth (increased latency) and low stress response (95). In total,  
334 the population obtained was homogenously composed of cells characterized as Viable

335 but non-Culturable cells (VBNC) (Figure 1), phenotype well-known in many bacteria and  
336 first described in 1982 in *Escherichia coli* (105). Among fungi, this phenotype has been  
337 described in *S. cerevisiae* (106), *Candida stellata* (107) and in *Brettanomyces bruxellensis*  
338 grown in wine synthetic medium and induced by sulfur dioxide (108). *C. neoformans*  
339 VBNCs were induced by hypoxia and nutrient deprivation and a proportion of them were  
340 able to be reactivated by the vitamin B5 (panthotenic acid) with a doubling number of  
341 culturable cells (Figure 2). Of note, it has been shown is specific model in *E. coli* that the  
342 VBNCs were potentially unable to reactivate (109). Pantothenic acid is known to play a  
343 role in the process of division (cell cycle) and in the quorum-sensing phenomenon (110)  
344 The use of diluted medium (which is poor in nutrients) to try to reactivate VBNCs was  
345 attempted, reflecting the observation that rich medium can be deleterious and induce  
346 death (111). Diluted medium did not lead to reactivation of more cells than rich medium,  
347 but rather, the cells that did reactivate exhibited faster growth and an increased doubling  
348 time compared to rich media. This cell phenotype induced by diluted medium has been  
349 called rewiring (95). Finally, based on large omics studies, the authors of this study were  
350 able to show that *C. neoformans* VBNCs harbored a decreased and specific metabolic  
351 activity based on phenotypic microarrays, transcriptome, secretome, and proteome  
352 analysis (95). Specifically, the fatty acid pathway was required for the maintenance and  
353 the viability of the VBNCs, and quorum sensing and mTor pathways seemed to play an  
354 important role in generating and/or maintaining the phenotype. Interestingly, acetyl CoA  
355 is a key precursor for both fatty acids and pantothenic acid, suggesting that regulation  
356 of acetyl CoA is a major factor for the generation of VBNCs (112). Based on these  
357 findings, a basic model of the evolution of *C. neoformans* yeast cells from logarithmic  
358 phase to dormancy and dead cells can be summarized as depicted in Figure 2.  
359 An analysis of the bulk population of yeasts maintained 8-days in nutrient deprivation  
360 and anaerobiosis to generate VBNCs identified another sub-population of yeasts that are  
361 still able and ready to grow on agar-rich medium. This population can be considered as  
362 persister cells. Persister cells have been described in a population of bacteria exposed  
363 to fungicidal antibiotics as the small proportion of bacteria able to tolerate spontaneously  
364 and stochastically lytic drugs via different mechanisms (113, 114). It has been shown that

365 persists and VBNCs can coexist in specific model of study in the bacteria *Vibrio*  
366 *vulnificus* (115). Persister cells have been described in *C. albicans* biofilm (116) and seem  
367 to play a role in recurrent infection in human oral candidiasis associated to natural biofilm  
368 (117). In VBNC-inducing conditions of *C. neoformans*, one can consider that remaining  
369 cells able to enter grow rapidly after long exposure of harsh conditions could be related  
370 to such persister cells, since a specific metabolism seems to occur compared to that of  
371 the VBNCs, which need a specific stimulus to grow again. This needs to be studied in  
372 details in future research.

373

## 374 **5. Relevance and unsolved questions**

375

376 The recent study highlighting the capacity of *C. neoformans* to switch to VBNCs can be  
377 viewed as a model to explore dormancy and metabolism in this organism and in  
378 pathogenic fungi in general. Indeed, this phenotype have still not been evidenced *per se*  
379 in human infection yet, but experimental conditions and the number of yeasts needed to  
380 obtain the demonstration is clearly not compatible with what can be recovered from the  
381 CSF of a patient with CM. The question of observing yeasts in the CSF of patients after  
382 7 days of induction therapy with a negative culture on regular medium obviously raise  
383 the question whether these yeasts are VBNCs. Being involved in clinical diagnosis, I have  
384 observed that the morphology of these non-culturable yeasts is abnormal and close to  
385 that observed in murine infection and called Drop Cn (see above) (8). Indeed, dead yeasts  
386 are known to persist and keep their intact shape, although different stainings can help  
387 differentiating them from regular and living yeasts (8). VBNCs, or at least part of the VBNC  
388 population, have proven to be reactivable by pantothenic acid, part of the demonstration  
389 that these cells are VBNC. The mechanism behind the specific reactivation is yet to be  
390 elucidated, but the fact that pantothenic acid (Vitamin B5) is a precursor of coenzyme A,  
391 which is an essential compound that participates in the metabolism of fatty acids,  
392 carbohydrates, and proteins through the formation of various active thioesters and  
393 promotes virulence and growth (118). Indeed, fatty acid have been shown to be critical  
394 in VBNCs (95). VBNCs obtained in *C. neoformans* can be considered as similar to that

395 obtained from bacteria or parasites as the definition relies on viability, culturability, and  
396 reactivation upon specific stimuli. Nevertheless, the condition allowing the generation of  
397 VBNCs and the stimuli that reactivate the population are different in different organisms.  
398 Among organisms, many common conditions of induction rely on stresses including  
399 starvation, low oxygen, low temperature, desiccation, or a combination of these. On the  
400 other hand, resuscitation conditions are extremely variable, such as increased nutrient  
401 availability, temperature modifications, addition of chemicals, or addition of hosts factors,  
402 depending on the organism (119).

403 The biology of dormancy in *C. neoformans* is a budding field and yet there are many more  
404 questions than answers. We still lack data on the effect of antifungal drugs on dormant  
405 yeasts cells because the experimental setting allowing demonstration of the effect or the  
406 absence of effect is not easy to implement in dormant cells that are intrinsically not  
407 cultivable. Indeed, turning on dormancy with some VBNC inductors that remain to be  
408 discovered would definitely aid in treating acute infection. On the other hand, inducing  
409 VBNCs could also be the cause of relapse by producing insensitivity to current antifungal  
410 strategies. These factors need to be addressed. Moreover, we have no data yet on the  
411 possible extension of the VBNC phenotype to clinical isolates of *C. neoformans* type VNI  
412 and to other phylogenetic lineages or species. There is a chance that all clinical isolates  
413 could have varying propensities to generate VBNCs, and so the impact on infection could  
414 be variable, as already shown for phenotypes of interaction with macrophages (8, 83).  
415 We are currently exploring the effect of the host on the induction and maintenance of  
416 VBNCs with regards to the level of activation of primary monocytes. One important  
417 aspect we are also currently exploring is the impact of VBNC metabolism on the  
418 physiology of the macrophages. Both studies aim to understand the interplay between  
419 host and fungal metabolisms, opening the way to discover specific pathways that could  
420 be modulated to push the system in one or the other direction (more killing or less  
421 proliferation of yeasts).

422

423

424 **6. Conclusions**



425 To summarize, *C. neoformans* is able to adapt fantastically to various environments,  
426 some of which are very drastic, such as 8 days in complete anaerobiosis and without  
427 extracellular nutrients available. *C. neoformans* uses strategies to resist these conditions.  
428 It is first perfectly able to enter quiescence in nutrient starvation conditions (stationary  
429 phase) or to be pushed into dormancy under additional anaerobiosis exposure. In vivo,  
430 one can imagine that VBNCs/dormant yeasts are most likely hidden in the innate immune  
431 cells for years before being able to reactivate and multiply either in the body of  
432 immunocompromised patients but also in the environment. This makes *C. neoformans*  
433 the first relevant pathogenic organism in which to study fungal dormancy and its role in  
434 the pathogenesis in humans.

435  
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445 outside of the scope of this review on *Pneumocystis* diagnosis  
446  
447

448 **Figures legends:**

449

450 **Figure 1: Morphology of quiescent (Stationary phase), dormant and dead *C.***  
451 ***neoformans* yeasts.** The reference strain H99 was used in all conditions. STAT:  
452 stationary phase (Yeast Peptone Dextrose, YPD, 22 hours with agitation 150 rpm) (17);  
453 VBNC (after incubation 8 days in anaerobiosis and nutrient deprivation) (97); DEAD:  
454 morphology of dead cells called DropCn including one or two vacuoles (17).

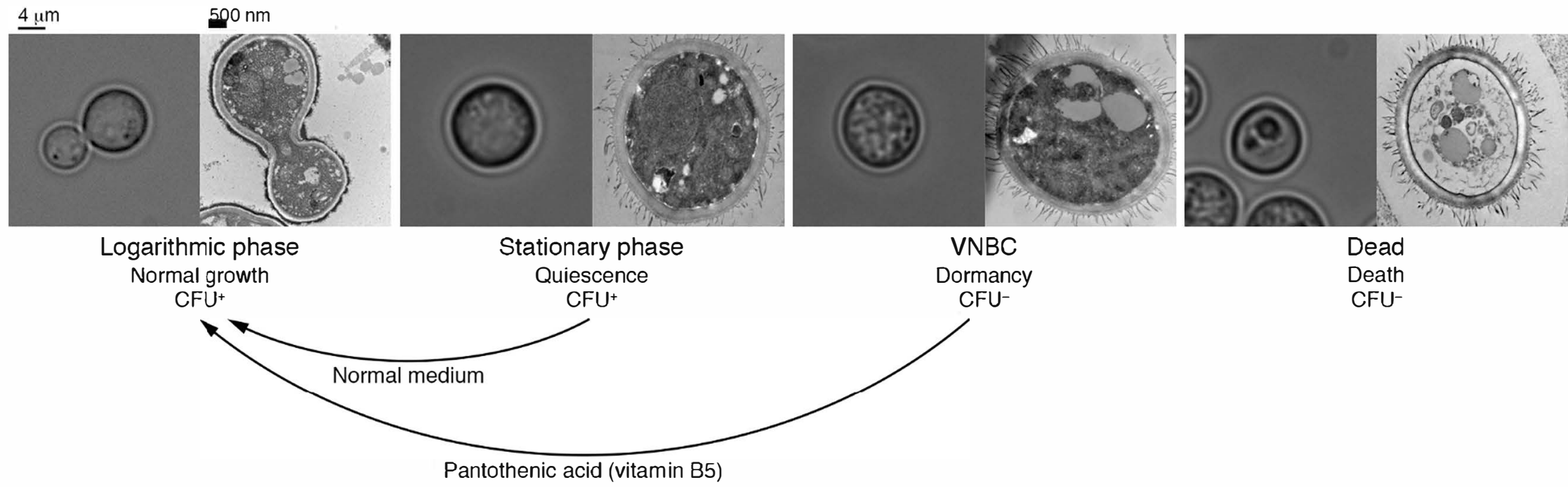
455

456 **Figure 2: Schematic representation of the evolution of *C. neoformans* phenotypes**  
457 **upon incubation in nutrient deprivation and anaerobiosis.** Yeasts cells under agitation  
458 and in glucose rich medium (YPD) are actively multiplying in logarithmic phase (LOG).  
459 Quiescent yeasts are culturable (Stationary phase, STAT) and does not need specific  
460 stimuli to grow in normal glucose rich (YPD) medium. Dormant yeasts (VBNC) are not  
461 culturable spontaneously and need a trigger stimulus for reactivation (addition of  
462 pantothenic acid). Dead yeasts (DEAD) are non-reversely unable to grow again (from 17,  
463 97).

464

465

Figure 2:



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