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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 μL . 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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441
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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).

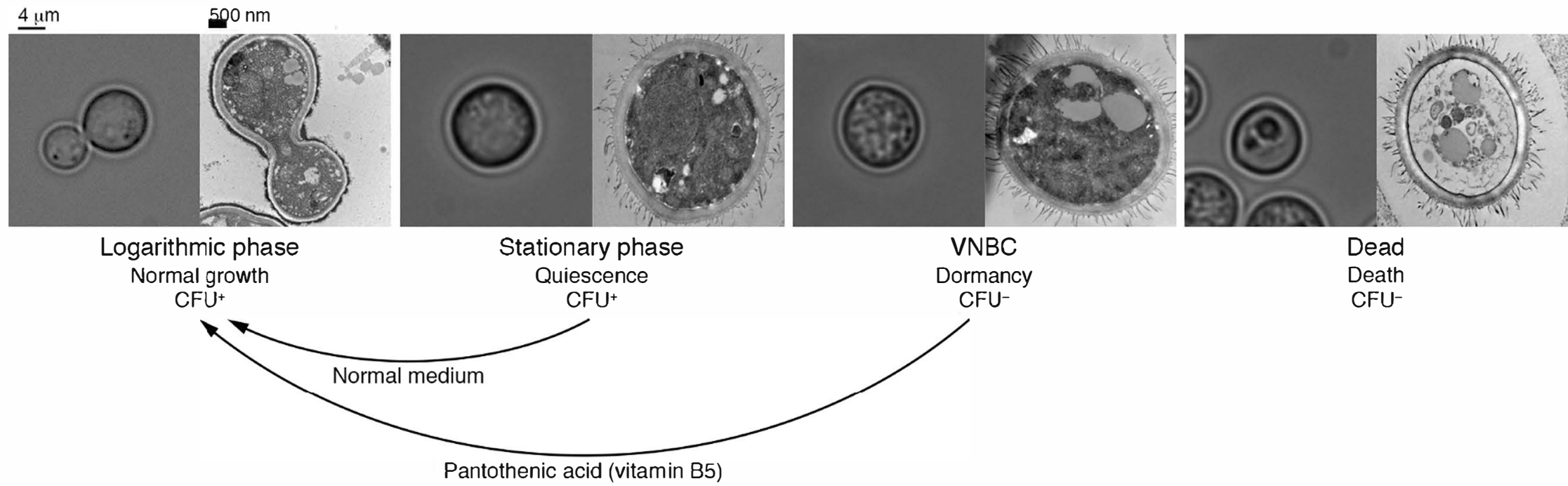
455

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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