Dormancy in Cryptococcus neoformans: 60 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.

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

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

Characteristics of the infection depend on both hosts and microbial factors. Fungal factors described as virulence factors influence the outcome of infection, according to data obtained in the mouse model of cryptococcosis (16), but also in vitro (17) and in humans (8, 18). Microbial adaptation to the hosts is complex and has been studied globally in lungs using histopathology (7) and global transcriptome analysis upon amoeba (19) and macrophage ingestion (20) or upon early infection of mice and rabbits (21, 22). Quiescence or dormancy is one of such adaptation that appears successful for enhancing the fungus's ability to survive, persist, reactivate, and then disseminate (23).

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

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

1. Natural history of cryptococcosis in humans

Cryptococcosis is one of the most frequent fungal invasive infection in humans worldwide (24). The vast majority of patients with cryptococcosis are HIV-positive patients, mostly those with CD4 T cells <100 cells per µL. Nevertheless, in western countries, the number of cryptococcosis cases recorded in HIV-negative individuals becomes higher than that in HIV-positive patients (25). Immunocompromised HIV-negative patients at risk of cryptococcosis are mainly solid organ transplant recipients, patients with systemic autoimmune disease, and those with hematological malignancies (26).

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

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

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

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

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

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

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

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

Early environmental exposure

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

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

Latency

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

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

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

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

Reactivation

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

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

2. The concept of dormancy in Fungi

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

Quiescence in *S. cerevisiae* has been studied for a long time. Recently, a strain of *S. cerevisiae* has been found "alive" in bottles of beer and Champagne from the 18th 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

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

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

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

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

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

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

but non-Culturable cells (VBNC) (Figure 1), phenotype well-known in many bacteria and first described in 1982 in *Escherichia coli* (105). Among fungi, this phenotype has been described in *S. cerevisiae* (106), *Candida stellata* (107) and in *Brettanomyces bruxellensis* grown in wine synthetic medium and induced by sulfur dioxide (108). *C. neoformans* VBNCs were induced by hypoxia and nutrient deprivation and a proportion of them were able to be reactivated by the vitamin B5 (panthotenic acid) with a doubling number of culturable cells (Figure 2). Of note, it has been shown is specific model in *E. coli* that the VBNCs were potentially unable to reactivate (109). Pantothenic acid is known to play a role in the process of division (cell cycle) and in the quorum-sensing phenomenon (110) The use of diluted medium (which is poor in nutrients) to try to reactivate VBNCs was attempted, reflecting the observation that rich medium can be deleterious and induce death (111). Diluted medium did not lead to reactivation of more cells than rich medium, but rather, the cells that did reactivate exhibited faster growth and an increased doubling time compared to rich media. This cell phenotype induced by diluted medium has been called rewiring (95). Finally, based on large omics studies, the authors of this study were able to show that *C. neoformans* VBNCs harbored a decreased and specific metabolic activity based on phenotypic microarrays, transcriptome, secretome, and proteome analysis (95). Specifically, the fatty acid pathway was required for the maintenance and the viability of the VBNCs, and quorum sensing and mTor pathways seemed to play an important role in generating and/or maintaining the phenotype. Interestingly, acetyl CoA is a key precursor for both fatty acids and pantothenic acid, suggesting that regulation of acetyl CoA is a major factor for the generation of VBNCs (112). Based on these findings, a basic model of the evolution of *C. neoformans* yeast cells from logarithmic phase to dormancy and dead cells can be summarized as depicted in Figure 2.

An analysis of the bulk population of yeasts maintained 8-days in nutrient deprivation and anaerobiosis to generate VBNCs identified another sub-population of yeasts that are still able and ready to grow on agar-rich medium. This population can be considered as persister cells. Persister cells have been described in a population of bacteria exposed to fungicidal antibiotics as the small proportion of bacteria able to tolerate spontaneously and stochastically lytic drugs via different mechanisms (113, 114). It has been shown that

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

5. Relevance and unsolved questions

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

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

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

6. Conclusions

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

Acknowledgments:
I would like to thank Françoise Dromer for her helpful comments and support and Stéphane Bretagne and Arturo Casadevall for their continuous support. No funding sources supported this work. In memory of my father for whom the question of "in between life and death" is not relevant anymore.

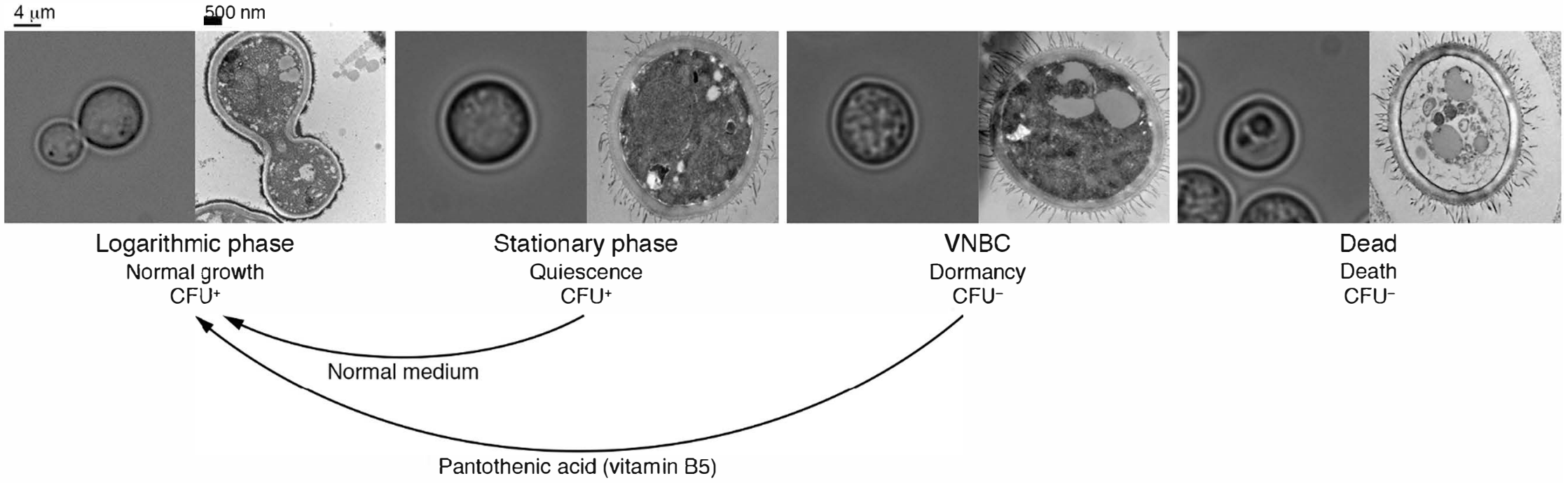
Competing interests: The author has declared that no competing interests exist.
Financial declaration: AA received a grant (102k€) from the ANR in 2019 as a partner in the French-Germany call to fight against antimicrobial resistance. AA owns a patent outside of the scope of this review on Pneumocystis diagnosis

Figures legends:

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

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

Figure 2:


4 µm
500 nm
Logarithmic phase
Normal growth
CFU+
Stationary phase
Quiescence
CFU+
VNBC
Dormancy
CFU−
Dead
Death
CFU−
Normal medium
Pantothenic acid (vitamin B5)

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