Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Microbiology

A titanic drug resistance threat in *Cryptococcus neoformans*

Hanna Zafar[1,3], Sophie Altamirano[2,3], Elizabeth R Ballou[1] and Kirsten Nielsen[2]

Increasing resistance to frontline antifungals is a growing threat to global health. In the face of high rates of relapse for patients with cryptococcal meningitis and frequent drug resistance in clinical isolates, recent insights into *Cryptococcus neoformans* morphogenesis and genome plasticity take on new and urgent meaning. Here we review the state of the understanding of mechanisms of drug resistance in the context of host-relevant changes in *Cryptococcus* morphology and cell ploidy.

Addresses
[1] Institute of Microbiology and Infection, School of Biosciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK
[2] Department of Microbiology and Immunology, University of Minnesota, Minneapolis, MN, 55455 USA

Corresponding authors: Ballou, Elizabeth R (e.r.ballou@bham.ac.uk), Nielsen, Kirsten (knielsen@umn.edu)
[3] Authors contributed equally.

Current Opinion in Microbiology 2019, **52**:158–164

This review comes from a themed issue on **Host-microbe interactions: fungi**

Edited by **Chad A Rappleye** and **Duncan Wilson**

For a complete overview see the Issue and the Editorial

Available online 22nd November 2019

https://doi.org/10.1016/j.mib.2019.11.001

1369-5274/

The increasing magnitude of antimicrobial resistance is a cause for global concern, particularly for fungal infections with few treatment options. Antifungal drug resistance affects not only worldwide population health, but also healthcare costs and gross domestic product (GDP) [1,2]. Development of drug resistance in the human fungal pathogen *Cryptococcus neoformans*, which causes an estimated one million symptomatic infections each year, could have a devastating impact on human health, yet only recently have studies begun to unravel the complex processes leading to antifungal drug resistance [3–6].

In 2010, a newly described morphotype, the *Cryptococcus* titan cell, was demonstrated to mediate pathogenesis [7–9]. Several reviews have discussed titan cell characteristics and mechanisms of formation [10,11]. Yet a recent study shows titan cells could also play a crucial role in stress adaptation and antifungal drug resistance [12]. Here, we examine the available literature that links antifungal drug resistance and the titan cell morphotype and urge improved understanding of these processes in patient outcome.

How does *C. neoformans* cause disease?

C. neoformans infection starts with inhalation of airborne fungal spores [13]. Within the lungs the spores can cause pulmonary infection, such as pneumonia, before disseminating to the central nervous system (CNS) to cause meningitis [14]. Cryptococcal meningitis is predominantly a disease of the immunocompromised – with organ transplant recipients, cancer chemotherapy patients, and individuals with advanced HIV at highest risk of developing cryptococcosis.

While the vast majority of *C. neoformans* patients present with meningitis, a number of reports have recently highlighted cryptococcal pneumonia as an underappreciated aspect of the disease [15–19]. While reports of pulmonary disease in immunocompromised individuals may be quite rare, asymptomatic or latent *C. neoformans* infection in the lungs of healthy people is common [20,21]. Serological studies show this initial pulmonary infection is acquired early in life, typically by five years of age [22,23]. Most adults have a *Cryptococcus* granuloma in their lungs [20,21,24–26], and strain genotyping of *C. neoformans* in immigrant populations show disease in immunocompromised individuals is due to strains acquired during childhood [27–30].

The impact of these asymptomatic pulmonary *C. neoformans* infections is unknown. Seropositivity has been linked to increased rates of asthma [31,32]. In addition, 20% of adult cryptococcosis patients are immunocompetent, suggesting unappreciated risk of progression to cryptococcal meningitis [11,33]. Most intriguing, these lifelong infections could have a dramatic impact on the development of drug resistance for patients that become immunocompromised and develop cryptococcosis later in life. Antifungal drug exposure throughout an individual's lifespan, either from environmental exposures due to agricultural antifungal use or during treatment of other fungal infections, could lead to drug resistance occurring before the individual presents with cryptococcosis. In a recent study in Uganda, Smith *et al.* noted increased resistance to the antifungal drug fluconazole in initial *C. neoformans* isolates from cryptococcal meningitis patients with advanced HIV. In the decade since antifungal drug use became widespread, over 30% of *C. neoformans* isolates are no longer susceptible to fluconazole [34]. A similar level of resistance was observed in Tanzanian clinical isolates, where 25% of initial cultures were resistant at time of diagnosis [35]. However, despite rising levels of resistance similar to those observed for other major fungal pathogens, there is reason to suspect that mechanisms of resistance may be distinct.

Mechanisms of antifungal drug resistance in *C. neoformans*

In the absence of successful treatment, cryptococcal meningitis is lethal. The standard of care requires a short course of amphotericin B in combination with flucytosine, followed by consolidation fluconazole monotherapy. Relapse has been observed in up to 60% of meningitis patients [36–41]. In cases of relapse, fluconazole resistance is frequently observed.

Mechanisms of antifungal drug resistance in fungi can be classified in two ways: heritable and transient. Heritable fluconazole resistance in *Candida albicans* and *Aspergillus* sp. is linked to point mutations, most commonly in the drug target *ERG11/CYP51* or drug transporters; *ERG11* single nucleotide polymorphisms (SNPs) alter drug-target interaction or lead to an increase in Erg11 protein levels, while gene duplications lead to increased multidrug transporter gene copy number [42–49]. However, while multiple studies have looked for *ERG11* point mutations in *C. neoformans*, these are rarely identified in patient isolates [4,35,50–53]. Other additional point mutations have been identified in mutator strains and other clinical isolates, but their exact contribution to drug resistance is yet to be determined [35,51,54–56].

In contrast, transient resistance is well documented in *C. neoformans*. *In vitro* and *in vivo* studies have revealed intrinsic hetero-resistance: transient resistance of a subpopulation of cells that are able to proliferate in the presence of high concentrations of fluconazole but lose their resistance once the drug stress is removed [41,57]. Genomic analyses have shown this hetero-resistance is mediated by aneuploidy of chromosomes 1, 4, 6, and 10 [12,35,57–59]. Interestingly, hetero-resistance can be suppressed by treating *C. neoformans* with fluconazole and flucytosine in combination [35].

As discussed above, during cryptococcosis, persistent antigenemia suggests that continued active infection may create a reservoir for the emergence of drug resistance during long-term therapy [22]. The emergence of this resistance was recently demonstrated *in vivo* in a cohort of HIV- infected Tanzanian patients [35]. Stone *et al.* found a fluconazole hetero-resistant subpopulation of up to 25% of colonies in certain clinical isolates that were able to grow in supra-MIC concentrations before any fluconazole treatment was given. Within seven days of fluconazole monotherapy, 11 out 13 patients (85%) had an increase in degree of hetero-resistance leading to an increase in MIC [35]. Combinatorial treatment with fluconazole and flucytosine was seen to supress the growth of hetero-resistant cells *in vitro* and *in vivo*. Although they saw continued suppression with this combination therapy *in vivo*, after 24 hours the hetero-resistant colonies reappeared *in vitro*. Interestingly, these newly grown hetero-resistant colonies were found to have developed additional resistance to flucytosine [35].

The mechanisms driving this process remain unclear, but several possibilities have been proposed based on *in vitro* studies. First, Altamirano *et al.*, demonstrated a direct impact for fluconazole on yeast cell nuclear division, consistent with findings in the ascomycete yeast *Candida albicans* [5,60,61]. The diploid *C. albicans* develops aneuploidy in the presence of fluconazole through production of intermediate tetraploid multimeras due to incomplete cytokinesis [61]. Similarly, *C. neoformans* was also found to produce multinucleate, incompletely septate cells in the presence of fluconazole [5]. Alternatively, Chang *et al.* demonstrated that fluconazole resistance may arise from an originating cell that is uninucleate and euploid, with aneuploidy arising through mis-segregation of chromosomes [6]. Together, this suggests that fluconazole may directly (through blocking cytokinesis) or indirectly (through genome plasticity) induce drug resistance.

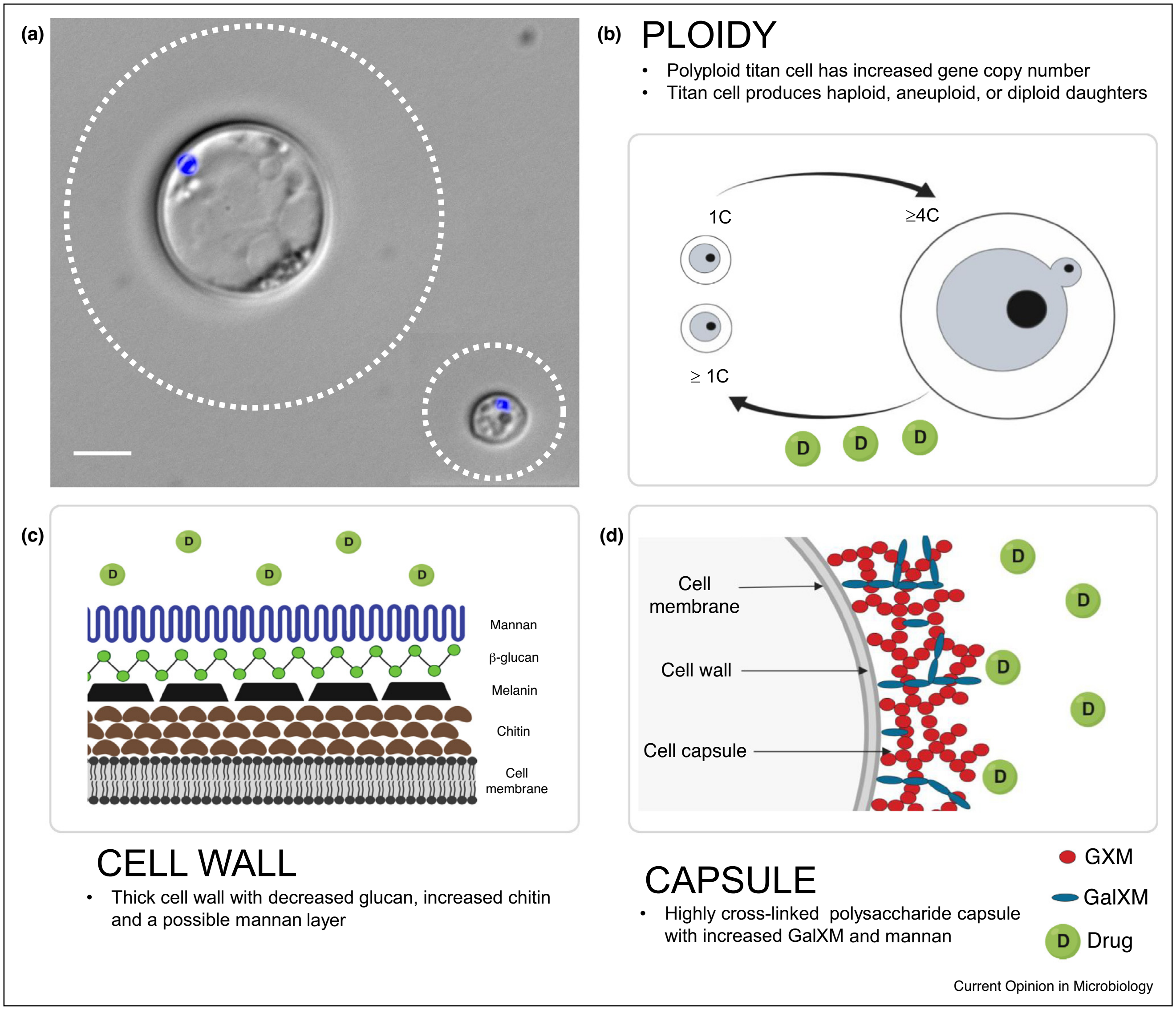
What is a titan cell and how does it influence antifungal drug resistance?

During the initial pulmonary infection, 20–30% of *C. neoformans* cells in the lungs convert into large polyploid titan cells [7,8]. Titan cells are observed in human samples [62,63] and in mouse models [7,8], and recent studies have identified *in vitro* culture conditions [64–66]. Titan cells are defined by their high ploidy ($\geq$4C), a cell body size larger than 10 μm in diameter, thick cell wall, and highly cross-linked capsule (Figure 1) [7,8,10].

Importantly, titan cells are critical for the overall virulence of *C. neoformans* and dissemination to the central nervous system [9]. Detailed analyses of the role of titan cells in pathogenesis have identified at least two distinct functions. First, structural changes associated with the titan cell, such as their increased cell size and modification of the cell wall and capsule structure, alter recognition by the host immune response [7,8,67–73]. This altered recognition not only promotes survival of the titan cell itself through reduced phagocytosis, but also shifts the adaptive immune response to promote *Cryptococcus* cell survival and dissemination to the central nervous system [70].

Second, and perhaps most intriguing in the context of drug resistance, is that titan cells generate daughter cells

Figure 1



Titan cell characteristics likely to impact drug resistance: **(a)** Cell size increase — Titan cells exhibit an increase in cell size that prevents phagocytosis and promotes survival. An increase in survival may provide additional opportunity for acquisition of drug resistance during infection. DIC image of a titan cell (left) and typical cell (right). Cells were isolated from the lungs of a mouse and stained with DAPI to visualize DNA (blue). White dashed lines outline capsules. Scale bar is 10 μm. **(b)** Ploidy – Titan cells have increased ploidy levels (≥4C) and produce aneuploid daughter cells (>1C) that are more resistant to the antifungal, fluconazole. Thus the increase in gene copy number in titan cells and their unique ability to undergo genome reductions may promote survival during drug treatment. **(c)** Cell wall and **(d)** Capsule — Compared to typical cells, titan cells have a thickened cell wall that contains increased chitin and a mannan fibril layer, and they have a more highly cross-linked capsule with increased GalXM and mannan. These cell surface alterations may reduce drug efficacy through decreased drug penetrance.

that exhibit increased resistance to fluconazole and may be better adapted to the host environment [12]. *Cryptococcus* cells are typically haploid, containing a single copy of the genome. Titan cells are polyploid with 4, 8, 16, and more copies of the genome [7,8,12,64,74]. Yet titan cells undergo a genome reduction when they produce daughter cells — often generating haploid, diploid, or aneuploid daughter cells [7,12,64]. Gerstein *et al.* showed that populations of daughter cells derived from titan cells are more resistant to oxidative and nitrosative stresses similar to those used by the host immune system [12]. Gerstein *et al.* also showed that *in vivo* derived titan cells incubated in the presence of fluconazole produce populations of daughter cells that are resistant to fluconazole, and whole genome sequencing revealed these daughter cells are aneuploid [12]. Thus, the titan cell morphotype may be a mechanism for the generation of the genomic plasticity underlying hetero-resistance and aneuploidy leading to drug resistance in *C. neoformans*.

Other mechanisms of resistance

In addition to contributing to fluconazole resistance through formation of aneuploid daughter cells, titan cells exhibit unique cell surface alterations that may also play a role in the development of drug resistance. The polysaccharide capsule surrounding the *C. neoformans* cell, which acts both as a protective outer layer and an immune modulatory compound, shows structural differences in titan cells. While typical-sized cells have diffuse capsules, the titan cell capsule is significantly denser and more highly crosslinked [8,72]. Additionally, titan cells have a cell wall up to 2−3 μm thick, compared to the 15−200 nm cell wall of typical-sized cells [10,68]. Analysis of titan cell walls in comparison to typical-sized cell walls revealed a decrease in glucan, an increase in chitin, and the possibility of a novel mannan layer on the surface of the cell wall [72]. It was recently shown that the increased chitin (a long-chain polymer of *N*-acetylglucosamine required for fungal cell wall rigidity) in the titan cell wall stimulates a detrimental Th2-mediated immune response [70]. There have not been any studies that have directly linked titan cell surface alterations with increased drug resistance, but several studies have shown both the capsule and cell wall to be important in conferring drug resistance. A recent review emphasized the potential for *in vivo* physiological changes in *C. neoformans*, such as titan cells and capsule enlargement, to act as barriers to effective treatment due to *in vitro* antifungal tests overlooking these factors [75]. Elegant work from the Fries lab has shown that old *C. neoformans* cells are more resistant to antifungals [76,77]. The increased drug resistance of old cells has been hypothesized to be due to the thickened cell wall of older cells, which could prevent or reduce drug penetration [76,77]. Studies have also shown that *C. neoformans* strains that produce larger capsules exhibit increased resistance to amphotericin B, which may also be due to reduced drug penetration [78–80]. Interestingly, both older cells with thickened cell walls and cells with larger capsules have also been shown to be more resistant to reactive oxygen species (ROS) induced by hydrogen peroxide stress, and both fluconazole and amphotericin B induce ROS in *C. neoformans* [75,77,78,81,82]. Taken together, the altered cell surface structure of titan cells could facilitate survival through reduction of the inhibitory effects of ROS during treatment. However, it has yet to be determined if a thickened cell wall is important in increased ROS resistance.

Conclusion

Titan cells are an important subpopulation of *C. neoformans* cells that arise early in infection and undergo unique morphological changes that contribute to increased virulence. Previous studies have highlighted the crucial role the cell surface alterations and enlarged size of titan cells play in evasion of the host immune system and dissemination to the brain. Additionally, it has been shown that titan cells exhibit increased ploidy levels and give rise to aneuploid daughter populations that are more stress resistant. However, the ability of titan cells to act as a source of adaptive phenotypic change to promote host adaptation and drug resistance still remains largely unexplored. Given the well-established importance of aneuploid formation in conferring fluconazole resistance in *C. neoformans*, the increased ploidy levels of titan cells and their ability to produce aneuploid daughter cells make investigating the contribution of titan cells to drug resistance especially intriguing. Given that titan cells are produced in the lungs during pulmonary infection, they may play a crucial role in the development of drug resistance during lifelong *Cryptococcus* infections. Future work examining the effects of titan cell formation on *in vivo* drug efficacy will help us better understand the mechanisms of drug resistance in *C. neoformans* and improve our current therapeutic and diagnostic approaches.

Funding

HZ is supported by an NC3Rs PhD studentship (NC/R001472/1). DSA is a TREM post-doctoral fellow supported by U.S. National Institutes of Health grant K12GM119955. ERB is supported by a Sir Henry Dale Fellowship jointly funded by the Wellcome Trust and the Royal Society (211241/Z/18/Z). KN is supported by U.S. National Institutes of Health grant R01AI134636.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

1. O'Neill J: **Tackling drug-resistance infections globally: a final report and recommendations**. *Review on Antimicrobial Resistance*. London, UK: Wellcome Trust; 2016 https://amr-review.org/.

2. Benedict K, Jackson BR, Chiller T, Beer KD: **Estimation of direct healthcare costs of fungal diseases in the United States**. *Clin Infect Dis* 2019, **68**:1791-1797 PMID: 30204844.

3. Semighini CP, Averette AF, Perfect JR, Heitman J: **Deletion of *Cryptococcus neoformans* AIF ortholog promotes chromosome aneuploidy and fluconazole-resistance in a metacaspase-independent manner**. *PLoS Pathog* 2011, **7** e1002364. PMID: 22114551.

4. Rhodes J, Beale MA, Vanhove M, Jarvis JN, Kannambath S, Simpson JA *et al.*: **A population genomics approach to assessing the genetic basis of within-host microevolution underlying recurrent cryptococcal meningitis infection**. *G3* 2017, **7**:1165-1176 PMID: 28188180.

5. Altamirano S, Fang D, Simmons C, Sridhar S, Wu P, Sanyal K *et al.*: **Fluconazole-induced ploidy change in *Cryptococcus neoformans* results from the uncoupling of cell growth and nuclear division**. *mSphere* 2017, **2**:e00205-17 PMID: 28630940

This *in vitro* work proposes a mechanism of fluconazole resistance in *Cryptococcus neoformans* through direct impact of fluconazole on yeast cell nuclear division.

6. Chang YC, Khanal Lamichhane A, Kwon-Chung KJ: ***Cryptococcus neoformans*, unlike *Candida albicans*, forms Aneuploid clones directly from uninucleated cells under fluconazole stress**. *mBio* 2018, **9**:e01290-18 PMID: 30514783

This study proposes another mechanism of fluconazole resistance with aneuploidy in *Cryptococcus neoformans* occurring through mis-segregation of chromosomes.

7. Okagaki LH, Strain AK, Nielsen JN, Charlier C, Baltes NJ, Chretien F *et al.*: **Cryptococcal cell morphology affects host cell interactions and pathogenicity**. *PLoS Pathog* 2010, **6** e1000953. PMID: 20585559

One of the first papers to observe titan cell formation as a morphotype of *C. neoformans* in mouse models.

8. Zaragoza O, Garcia-Rodas R, Nosanchuk JD, Cuenca-Estrella M, Rodriguez-Tudela JL, Casadevall A: **Fungal cell gigantism during mammalian infection**. *PLoS Pathog* 2010, **6** e1000945. PMID: 20585557

One of the first papers to observe Titan cell formation as a morphotype of *Cryptococcus neoformans* in mouse models.

9. Crabtree JN, Okagaki LH, Wiesner DL, Strain AK, Nielsen JN, Nielsen K: **Titan cell production enhances the virulence of *Cryptococcus neoformans***. *Infect Immun* 2012, **80**:3776-3785 PMID: 22890995

This study shows the importance of titan cells in promoting *C. neoformans* virulence.

10. Zaragoza O, Nielsen K: **Titan cells in *Cryptococcus neoformans*: cells with a giant impact**. *Curr Opin Microbiol* 2013, **16**:409-413 PMID: 23588027.

11. Zhou X, Ballou ER: **The *Cryptococcus neoformans* titan cell: from *in vivo* phenomenon to *in vitro* model**. *J Curr Clin Microbiol Rep* 2018, **5**:252-260.

12. Gerstein AC, Fu MS, Mukaremera L, Li Z, Ormerod KL, Fraser JA *et al.*: **Polyploid titan cells produce haploid and aneuploid progeny to promote stress adaptation**. *mBio* 2015, **6**:e01340-15 PMID: 26463162

This work shows *in vivo* derived titan cells are more resistant to various stresses, including fluconazole, and can produce aneuploid daughter cell populations that are also more fluconazole resistant, suggesting a role for titan cells in host adaptation.

13. Botts MR, Hull CM: **Dueling in the lung: how *Cryptococcus* spores race the host for survival**. *Curr Opin Microbiol* 2010, **13**:437-442 PMID: 20570552.

14. Casadevall A, Perfect JR: ***Cryptococcus neoformans***. Washington, DC: ASM Press; 1998.

15. Scriven JE, Botha FC, Schutz C, Lalloo DG, Wainwright H, Meintjes G: **Paradoxical respiratory failure due to cryptococcal pneumonia after amphotericin B treatment for HIV-associated cryptococcal meningitis**. *Med Mycol Case Rep* 2018, **19**:38-40 PMID: 29379704.

16. Wong ML, Back P, Candy G, Nelson G, Murray J: **Cryptococcal pneumonia in African miners at autopsy**. *Int J Tuberculosis Lung Dis* 2007, **11**:528-533 PMID: 17439676.

17. Spec A, Olsen MA, Raval K, Powderly WG: **Impact of infectious diseases consultation on mortality of cryptococcal infection in patients without HIV**. *Clin Infect Dis* 2017, **64**:558-564 PMID: 27927865.

18. Singh N, Alexander BD, Lortholary O, Dromer F, Gupta KL, John GT *et al.*: ***Cryptococcus neoformans* in organ transplant recipients: impact of calcineurin-inhibitor agents on mortality**. *J Infect Dis* 2007, **195**:756-764 PMID: 17262720.

19. Raval K, Powderly W, Spec A: **Presentation and outcome of cryptococcal infection varies by predisposing illness**. *Open Forum Infect Dis* 2017, **49(Suppl. 1)**:S77-S78.

20. Baker RD: **The primary pulmonary lymph node complex of Cryptococcosis**. *Am J Clin Pathol* 1976, **65(1)**:83-92 PMID: 1246992.

21. Baker RD, Haugen RK: **Tissue changes and tissue diagnosis in cryptococcosis; a study of 26 cases**. *Am J Clin Pathol* 1955, **25**:14-24 PMID: 14349908.

22. Goldman DL, Khine H, Abadi J, Lindenberg DJ, Pirofski L, Niang R *et al.*: **Serologic evidence for *Cryptococcus neoformans* infection in early childhood**. *Pediatrics* 2001, **107**:E66 PMID: 11331716.

23. Casadevall A, Cleare W, Feldmesser M, Glatman-Freedman A, Goldman DL, Kozel TR *et al.*: **Characterization of a murine monoclonal antibody to *Cryptococcus neoformans* polysaccharide that is a candidate for human therapeutic studies**. *Antimicrob Agents Chemother* 1998, **42**:1437-1446 PMID: 9624491.

24. Cameron ML, Bartlett JA, Gallis HA, Waskin HA: **Manifestations of pulmonary cryptococcosis in patients with acquired immunodeficiency syndrome**. *Rev Infect Dis* 1991, **13**:64-67 PMID: 2017634.

25. Paterson DL, Singh N, Gayowski T, Marino IR: **Pulmonary Nodules in liver transplant recipients**. *Medicine* 1998, **77**:50-58 PMID: 9465863.

26. Rolston KV, Rodriguez S, Dholakia N, Whimbey E, Raad I: **Pulmonary infections mimicking cancer: a retrospective, three-year review**. *Support Care Cancer* 1997, **5**:90-93 PMID: 9069606.

27. Saha DC, Goldman DL, Shao X, Casadevall A, Husain S, Limaye AP *et al.*: **Serologic evidence for reactivation of cryptococcosis in solid-organ transplant recipients**. *Clin Vaccine Immunol* 2007, **14**:1550-1554 PMID: 17959819.

28. Dromer F, Casadevall A, Perfect J, Sorrell T: ***Cryptococcus neoformans*: latency and disease**. In *Cryptococcus: From Human Pathogen to Model Yeast.* Edited by Heitman H, Kozel TR, Kwon-Chung KJ, Perfect JR, Casadevall A. Washington, DC, USA: ASM Press; 2011.

29. Abadi J, Pirofski L: **Antibodies reactive with the cryptococcal capsular polysaccharide glucuronoxylomannan are present in sera from children with and without human immunodeficiency virus infection**. *J Infect Dis* 1999, **180**:915-919 PMID: 10438394.

30. Garcia-Hermoso D, Janbon G, Dromer F: **Epidemiological evidence for dormant *Cryptococcus neoformans* infection**. *J Clin Microbiol* 1999, **37**:3204-3209 PMID: 10488178.

31. Grahnert A, Muller U, von Buttlar H, Treudler R, Alber G: **Analysis of asthma patients for cryptococcal seroreactivity in an urban German area**. *Med Mycol* 2015, **53**:576-586 PMID: 26026172.

32. Goldman DL, Davis J, Bommarito F, Shao X, Casadevall A: **Enhanced allergic Inflammation and airway responsiveness in rats with chronic *Cryptococcus neoformans* infection: potential role for fungal pulmonary infection in the pathogenesis of asthma**. *J Infect Dis* 2006, **193**:1178-1186 PMID: 16544260.

33. Day JN, Hoang TN, Duong AV, Hong CT, Diep PT, Campbell JI *et al.*: **Most cases of cryptococcal meningitis in HIV-Uninfected patients in vietnam are due to a distinct amplified fragment length polymorphism-defined cluster of *Cryptococcus neoformans* var. *grubii* VN1**. *J Clin Microbiol* 2011, **49**:658-664 PMID: 21159929.

34. Smith KD, Achan B, Hullsiek KH, McDonald TR, Okagaki LH, Alhadab AA *et al.*: **Increased antifungal drug resistance in clinical isolates of *Cryptococcus neoformans* in Uganda**. *Antimicrob Agents Chemother* 2015, **59**:7197-7204 PMID: 26324276.

35. Stone NR, Rhodes J, Fisher MC, Mfinanga S, Kivuyo S, Rugemalila J *et al.*: **Dynamic ploidy changes drive fluconazole resistance in human cryptococcal meningitis**. *J Clin Investig* 2019, **129**:999-1014 PMID: 30688656

This is the first study to describe the occurrence of heteroresistant, aneuploid populations of *C. neoformans* in human samples and show the increase of heteroresistance over the course of fluconazole monotherapy.

36. Perfect JR, Dismukes WE, Dromer F, Goldman DL, Graybill JR, Hamill RJ *et al.*: **Clinical practice guidelines for the management of cryptococcal disease: 2010 update by the**

infectious diseases society of America. *Clin Infect Dis* 2010, **50**:291-322 PMID: 20047480.

37. Bicanic T, Harrison T, Niepieklo A, Dyakopu N, Meintjes G: **Symptomatic relapse of HIV-associated cryptococcal meningitis after initial fluconazole monotherapy: the role of fluconazole resistance and immune reconstitution**. *Clin Infect Dis* 2006, **43**:1069-1073 PMID: 16983622.

38. Mpoza E, Rhein J, Abassi M: **Emerging fluconazole resistance: implications for the management of cryptococcal meningitis**. *Med Mycol Case Rep* 2018, **19**:30-32 PMID: 29234588.

39. Bongomin F, Oladele RO, Gago S, Moore CB, Richardson MD: **A systematic review of fluconazole resistance in clinical isolates of *Cryptococcus* species**. *Mycoses* 2018, **61**:290-297 PMID: 29377368.

40. Sar B, Monchy D, Vann M, Keo C, Sarthou JL, Buisson Y: **Increasing *in vitro* resistance to fluconazole in *Cryptococcus neoformans* Cambodian isolates: April 2000 to March 2002**. *J Antimicrob Chemother* 2004, **54**:563-565 PMID: 15254027.

41. Xu J, Onyewu C, Yoell HJ, Ali RY, Vilgalys RJ, Mitchell TG: **Dynamic and heterogeneous mutations to fluconazole resistance in *Cryptococcus neoformans***. *Antimicrob Agents Chemother* 2001, **45**:420-427 PMID: 11158735.

42. Lupetti A, Danesi R, Campa M, Del Tacca M, Kelly S: **Molecular basis of resistance to azole antifungals**. *Trends Mol Med* 2002, **8**:76-81 PMID: 11815273.

43. White TC, Holleman S, Dy F, Mirels LF, Stevens DA: **Resistance mechanisms in clinical isolates of *Candida albicans***. *Antimicrob Agents Chemother* 2002, **46**:1704-1713 PMID: 12019079.

44. Perea S, Lopez-Ribot JL, Kirkpatrick WR, McAtee RK, Santillan RA, Martinez M *et al.*: **Prevalence of molecular mechanisms of resistance to azole antifungal agents in *Candida albicans* strains displaying high-level fluconazole resistance isolated from human immunodeficiency virus-infected patients**. *Antimicrob Agents Chemother* 2001, **45**:2676-2684 PMID: 11557454.

45. Xiang M-J, Wei B, Shi C, Liu J-Y, Ni P-H, Wang S *et al.*: **Erg11 mutations associated with azole resistance in clinical isolates of *Candida albicans***. *FEMS Yeast Res* 2013, **13**:386-393 PMID: 23480635.

46. Flowers SA, Colon B, Whaley SG, Schuler MA, Rogers PD: **Contribution of clinically derived mutations in ERG11 to azole resistance in *Candida albicans***. *Antimicrob Agents Chemother* 2015, **59**:450-460 PMID: 25385095.

47. Wang B, Huang L-H, Zhao J-X, Wei M, Fang H, Wang D-Y *et al.*: **ERG11 mutations associated with azole resistance in *Candida albicans* isolates from vulvovaginal candidosis patients**. *Asian Pac J Trop Biomed* 2015, **5**:909-914.

48. Mann PA, Parmegiani RM, Wei SQ, Mendrick CA, Li X, Loebenberg D *et al.*: **Mutations in *Aspergillus fumigatus* resulting in reduced susceptibility to posaconazole appear to be restricted to a single amino acid in the cytochrome P450 14alpha-demethylase**. *Antimicrob Agents Chemother* 2003, **47**:577-581 PMID: 12543662.

49. Rybak JM, Ge W, Wiederhold NP, Parker JE, Kelly SL, Rogers PD *et al.*: **Mutations in *hmg1*, challenging the paradigm of clinical triazole resistance in *Aspergillus fumigatus***. *mBio* 2019, **10**: e00437-19 PMID: 30940706.

50. Arras SDM, Ormerod KL, Erpf PE, Espinosa MI, Carpenter AC, Blundell RD *et al.*: **Convergent microevolution of *Cryptococcus neoformans* hypervirulence in the laboratory and the clinic**. *Sci Rep* 2017, **7**:17918 PMID: 29263343.

51. Boyce KJ, Wang Y, Verma S, Shakya VPS, Xue C, Idnurm A: **Mismatch repair of DNA replication errors contributes to microevolution in the pathogenic fungus *Cryptococcus neoformans***. *mBio.* 2017, **8**:e00595-17 PMID: 28559486.

52. Rodero L, Mellado E, Rodriguez AC, Salve A, Guelfand L, Cahn P *et al.*: **G484S amino acid substitution in Lanosterol 14-α demethylase (ERG11) Is related to fluconazole resistance in a recurrent *Cryptococcus neoformans* clinical isolate**. *Antimicrob Agents Chemother* 2003, **47**:3653-3656 PMID: 14576140.

53. Sionov E, Chang YC, Garraffo HM, Dolan MA, Ghannoum MA, Kwon-Chung KJ: **Identification of a *Cryptococcus neoformans* cytochrome P450 lanosterol 14alpha-demethylase (Erg11) residue critical for differential susceptibility between fluconazole/voriconazole and itraconazole/posaconazole**. *Antimicrob Agents Chemother* 2012, **56**:1162-1169 PMID: 22155829.

54. Chow EW, Morrow CA, Djordjevic JT, Wood IA, Fraser JA: **Microevolution of *Cryptococcus neoformans* driven by massive tandem gene amplification**. *Mol Biol Evol* 2012, **29**:1987-2000 PMID: 22334577.

55. Liu OW, Chun CD, Chow ED, Chen C, Madhani HD, Noble SM: **Systematic genetic analysis of virulence in the human fungal pathogen *Cryptococcus neoformans***. *Cell* 2008, **135**:174-188 PMID: 18854164.

56. Magditch DA, Liu TB, Xue C, Idnurm A: **DNA Mutations mediate microevolution between host-adapted forms of the pathogenic fungus *Cryptococcus neoformans***. *PLoS Pathog* 2012, **8** e1002936. PMID: 23055925.

57. Sionov E, Chang YC, Garraffo HM, Kwon-Chung KJ: **Heteroresistance to fluconazole in *Cryptococcus neoformans* is intrinsic and associated with virulence**. *Antimicrob Agents Chemother* 2009, **53**:2804-2815 PMID: 19414582
This paper defines fluconazole heteroresistance in *C. neoformans* and correlates fluconazole heteroresistance with virulence.

58. Sionov E, Lee H, Chang YC, Kwon-Chung KJ: ***Cryptococcus neoformans* overcomes stress of azole drugs by formation of disomy in specific multiple chromosomes**. *PLoS Pathog* 2010, **6** e1000848. PMID: 20368972
This *in vitro* study highlights the important role of aneuploid formation in fluconazole heteroresistance in *C. neoformans*.

59. Ngamskulrungroj P, Chang Y, Hansen B, Bugge C, Fischer E, Kwon-Chung KJ: **Characterization of the chromosome 4 genes that affect fluconazole-induced disomy formation in *Cryptococcus neoformans***. *PloS One* 2012, **7** e33022. PMID: 22412978.

60. Selmecki AM, Dulmage K, Cowen LE, Anderson JB, Berman J: **Acquisition of aneuploidy provides increased fitness during the evolution of antifungal drug resistance**. *PLoS Genet* 2009, **5** e1000705. PMID: 19876375.

61. Harrison BD, Hashemi J, Bibi M, Pulver R, Bavli D, Nahmias Y *et al.*: **A tetraploid intermediate precedes aneuploid formation in yeasts exposed to fluconazole**. *PLoS Biol* 2014, **12** e1001815. PMID: 24642609.

62. Cruickshank JG, Cavill R, Jelbert M: ***Cryptococcus neoformans* of unusual morphology**. *Appl Microbiol* 1973, **25**:309-312 PMID: 4121033.

63. Love GL, Boyd GD, Greer DL: **Large *Cryptococcus neoformans* isolated from brain abscess**. *J Clin Microbio* 1985, **22**:1068-1070 PMID: 3905847.

64. Dambuza IM, Drake T, Chapuis A, Zhou X, Correia J, Taylor-Smith L *et al.*: **The *Cryptococcus neoformans* Titan cell is an inducible and regulated morphotype underlying pathogenesis**. *PLoS Pathog* 2018, **14** e1006978. PMID: 29775474
One of three back-to-back studies that describes a method of Titan cells induction *in vitro* which could help answer questions about the molecular mechanism of drug resistance in Titan cells.

65. Hommel B, Mukaremera L, Cordero RJB, Coelho C, Desjardins CA, Sturny-Leclere A *et al.*: **Titan cells formation in *Cryptococcus neoformans* is finely tuned by environmental conditions and modulated by positive and negative genetic regulators**. *PLoS Pathog* 2018, **14** e1006982. PMID: 29775480
One of three back-to-back studies that describes a method of Titan cells induction *in vitro* which could help answer questions about the molecular mechanism of drug resistance in Titan cells.

66. Trevijano-Contador N, de Oliveira HC, García-Rodas R, Rossi SA, Llorente I, Zaballos Á *et al.*: ***Cryptococcus neoformans* can form titan-like cells *in vitro* in response to multiple signals**. *PLoS Pathog* 2018, **14** e1007007. PMID: 29775477

One of three back to back studies that describes a method of Titan cells induction *in vitro* which could help answer questions about the molecular mechanism of drug resistance in Titan cells.

67. Okagaki LH, Nielsen K: **Titan cells confer protection from phagocytosis in *Cryptococcus neoformans* infections**. *Eukaryot Cell* 2012, **11**:820-826 PMID: 22544904.

68. Wiesner DL, Specht CA, Lee CK, Smith KD, Mukaremera L, Lee ST *et al.*: **Chitin recognition via chitotriosidase promotes pathologic type-2 Helper T cell responses to cryptococcal infection**. *PLoS Patho* 2015, **11** e1004701. PMID: 25764512.

69. Evans RJ, Li Z, Hughes WS, Djordjevic JT, Nielsen K, May RC: **Cryptococcal phospholipase B1 is required for intracellular proliferation and control of titan cell morphology during macrophage infection**. *Infect Immun* 2015, **83**:1296-1304 PMID: 25605772.

70. Wiesner DL, Smith KD, Kotov DI, Nielsen JN, Bohjanen PR, Nielsen K: **Regulatory T cell induction and retention in the lungs drives suppression of detrimental type 2 Th cells during pulmonary cryptococcal infection**. *J Immunol* 2016, **196**:365-374 PMID: 26590316.

71. Mukaremera L, Nielsen K: **Adaptive immunity to *Cryptococcus neoformans* infections**. *J Fungi* 2017, **3**:64 PMID: 29333430.

72. Mukaremera L, Lee KK, Wagener J, Wiesner DL, Gow NAR, Nielsen K: **Titan cell production in *Cryptococcus neoformans* reshapes the cell wall and capsule composition during infection**. *Cell Surf* 2018, **1**:15-24 PMID: 30123851

This paper describes the differences in the cell wall and capsule of *in vivo* titan cells compared to *in vivo* and *in vitro* typical-sized cells.

73. Probert M, Zhou X, Goodall M, Johnston SA, Bielska E, Ballou ER *et al.*: **A glucuronoxylomannan epitope exhibits serotype-specific accessibility and redistributes towards the capsule surface during titanization of the fungal pathogen *Cryptococcus neoformans***. *Infect Immun* 2019, **87**:e00731-18 PMID: 30670549.

74. Alanio A, Vernel-Pauillac F, Sturny-Leclere A, Dromer F: ***Cryptococcus neoformans* host adaptation: toward biological evidence of dormancy**. *mBio* 2015, **6**:e02580-14 PMID: 25827423.

75. Grossman NT, Casadevall A: **Physiological differences in *Cryptococcus neoformans* strains *in vitro* versus *in vivo* and their effects on antifungal susceptibility**. *Antimicrob Agents Chemother* 2017, **61**:e02108-16 PMID: 28031206.

76. Jain N, Cook E, Xess I, Hasan F, Fries D, Fries BC: **Isolation and characterization of senescent *Cryptococcus neoformans* and implications for phenotypic switching and pathogenesis in chronic cryptococcosis**. *Eukaryot Cell* 2009, **8**:858-866 PMID: 19411622.

77. Bouklas T, Pechuan X, Goldman DL, Edelman B, Bergman A, Fries BC: **Old *Cryptococcus neoformans* cells contribute to virulence in cchronic cryptococcosis**. *mBio* 2013, **4**:e00455-13 PMID: 23943761.

78. Zaragoza O, Chrisman CJ, Castelli MV, Frases S, Cuenca-Estrella M, Rodriguez-Tudela JL *et al.*: **Capsule enlargement in *Cryptococcus neoformans* confers resistance to oxidative stress suggesting a mechanism for intracellular survival**. *Cell Microbiol* 2008, **10**:2043-2057 PMID: 18554313.

79. Vitale RG, Pascuccelli V, Afeltra J: **Influence of capsule size on the *in vitro* activity of antifungal agents against clinical *Cryptococcus neoformans* var. *grubii* strains**. *J Med Microbiol* 2012, **61**:384-388 PMID: 22074850.

80. Cordoba S, Afeltra J, Vitale RG: **Evaluation of the *in vitro* activity of Amphotericin B by time-kill curve methodology against large and small capsulate *C. neoformans* isolates**. *Diagn Microbiol Infect Dis* 2011, **71**:260-262 PMID: 21917396.

81. Peng CA, Gaertner AAE, Henriquez SA, Fang D, Colon-Reyes RJ, Brumaghim JL *et al.*: **Fluconazole induces ROS in *Cryptococcus neoformans* and contributes to DNA damage *in vitro***. *PLoS One* 2018, **13** e0208471. PMID: 30532246.

82. Mesa-Arango AC, Trevijano-Contador N, Roman E, Sanchez-Fresneda R, Casas C, Herrero E *et al.*: **The production of reactive oxygen species is a universal action mechanism of amphotericin B against pathogenic yeasts and contributes to the fungicidal effect of this drug**. *Antimicrob Agents Chemother* 2014, **58**:6627-6638 PMID: 25155595.