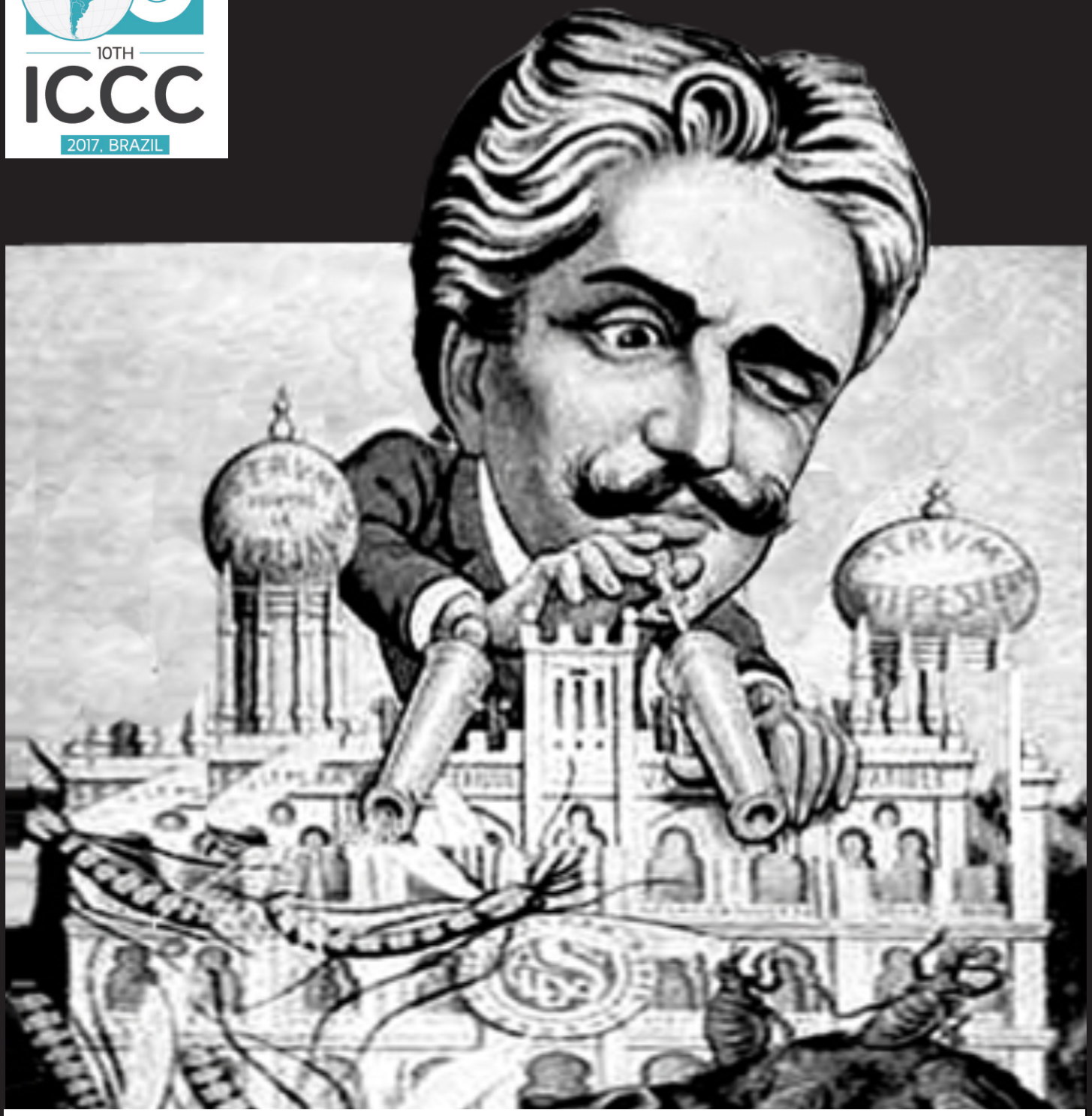
10TH ICCC 2017, BRAZIL

10th International Conference on *Cryptococcus* and Cryptococcosis



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INI Instituto Nacional de Infectologia Evandro Chagas

SBM Sociedade Brasileira de Microbiologia

CDTS Centro de Desenvolvimento Tecnológico em Saúde

CNPq Conselho Nacional de Desenvolvimento Científico e Tecnológico

CAPES

Burroughs Wellcome Fund

ISHAM International Society for Human and Animal Mycology

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LECTURERS' ABSTRACTS

Geographical dispersion of *Cryptococcus gattii* in Latin America

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The *Cryptococcus gattii* species complex now being recognized as an emerging fungal pathogen both in humans and animals, having the capacity to expand its geographical range and ecological niches.Even it is not the main causative agent of cryptococcosis worldwide, the emergence of new cases, especially since the 1999 ongoing outbreak in British Columbia, which is spreading to the Pacific Northwest of the United States, has called the attention of different research groups. Clinical cases due to *C. gattii* have been documented since 1961 in Latin America, where the first isolate typed further as VGIV, was recovered from a CSF sample obtained in a 38 year-old male Mexican patient.Subsequently, sporadic cases of the disease caused by this species have been reported in Argentina, Peru, Cuba and Venezuela.In 2003, a multicenter study, performed in collaboration with the University of Sydney, with Ibero-American isolates (clinical and environmental) recovered in Argentina (n=2), Brazil (n=6), Colombia (n=13), Guatemala (n=1), Mexico (n=19), Peru (n=1) and Venezuela (n=4),revealed that 20% of the isolates belonged to *C. gattii*. Very few cases of cryptococcosis due to *C. gattii* in animals have also been reported in the region, being documented from Aruba (goat), Brazil (parrots) and Cuba (cheetah).Latin American research groups have applied molecular typing, providing information on the distribution of the four well-known patterns, VGI-VGIV. These studies have revealed that molecular types VGII and VGIII are the most prevalent. More complex molecular techniques (AFLP, MLST and WGS)have allowed to study more profoundly the population structure of these isolates, demonstrating that the outbreaks reported on Vancouver Island and the Pacific Northwest arose from a highly-recombinant *C. gattii* AFLP6/VGII population in Northern Brazil, and that VGIII Mexican isolates, which are emerging as cause of disease in otherwise healthy patients, are most likely being the origin of the serotype B VGIII population, and Colombian isolates are the possible origin of the serotype C VGIII population. The distribution of *C. gattii* in the environment has also called the attention of Latin American research groups, especially in Brazil and Colombia, whose participants have conducted extensive and sustained environmental studies. Pioneering findings began in Brazil in the mid 90s, when Marcia Lazera's team identified hollows of trees as important reservoirs of the fungus, after continuously isolating the yeast from specimens in the Amazon, followed by a series of positive findings in numerous geographical diverse areas of the country. Colombia has also shown its stronghold in environmental studies, providing a wealth of data on the distribution of *C. gattii* in the environment.Product of these extensive samplings throughout the country is the first report on the recovery of serotype *C* from almond tree detritus in an endemic area for *C. gattii*.Recently, the information that has been collected during the environmental studies performed in Colombia since 1998 was used to build and test an ecological niche model for *C. gattii*, which predicted that this species is highly adaptive to different ecological conditions in the country. Sporadic findings have been reported in Argentina from sources like Eucalyptus and Tipuanatipu trees, and an exclusive report in Uruguay in 1989 from the nest of Polybiaoccidentalis. Molecular analysis of clinical isolates and environmental strains demonstrated the prevalence of VGIII in the region, reinforcing a more detailed population analysis of isolates from even broader geographical origins. Population genetic analysis putting the Latin American *C. gattii* isolates into a global context has shown at least for VGII that there have been multiple dispersals out of South America to different parts of the world.

Epidemiology of *Cryptococcus neoformans* in Latin America

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Latin America is a geographical and cultural area that includes about 30 countries, of which less than 50% have notification of cryptococcosis. According to the Pan American Health Organization prior to the emergence of antiretroviral treatment, 5-8% of HIV patients in developed countries had disseminated cryptococcosis. The incidence has progressively decreased and most cases occur in individuals with CD4 counts below 50 cells / mm3. This small talk will present the results obtained through an electronic search in Latin American countries for demographic, medical data (clinic, diagnosis and treatment) of patients diagnosed with cryptococcosis, as well as the microbiological data of Cryptococcus neoformans, the main etiological agent of this neuroinfection. The research was conducted in Latindex® (Regional Information System Online), Imbiomed® (Mexican Index of Latin American Biomedical Journals), Medigraphic® (Latin American Medical Journals Index), SciELO (Scientific Electronic Library Online) and PubMed®: Medline®. The criteria used were to put in a Web Browser, the word cryptococcosis with the word that indicates the name of the investigated country. In general, the data obtained show that Argentina, Brazil, Colombia and Mexico are Latin American countries where epidemiological investigation of cryptococcosis has been important. Also, as in other regions of the world, cryptococcosis mostly affects people: 1) male, 2) between 20 and 40 years old, 3) AIDS patients. Regarding the etiology, C. neoformans genotype VNI, sexual type "", presents in more than 90% of cases of cryptococcosis. Latin America is an important region in the prevalence of cryptococcosis related to AIDS patients and where the use of antiretroviral therapy has apparently decreased the incidence of infection. However, a characteristic in Latin American countries is that the lethality rate of cryptococcosis remains high.

The capsule architecture and its role in the pathogenesis of *Cryptococcus neoformans*

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Fungal sistemicinfections have become a major threat especially affecting individuals with a compromised immune system. One such infection is the cryptococcosis, a disease caused by *Cryptococcus neoformans* and *Cryptococcus gattii.* The main virulence factor, which produces deleterious effects on the immune system, is the polysaccharide (PS) capsule. This structure provides protection against mammalian hosts. In recent years, our group try to understand the characteristics that separate pathogenic and non-pathogenic cryptococcal species to determine the virulence potential of several members of this genus. Such perceptions may be important to anticipate the emergence of new virulence fungi consequence of global warming that could promote an increment of thermal tolerance of environmental fungal species, making these viable to adapt to mammalian temperatures and promote their interaction with human host. Our group characterized the similarities and differences between the capsules of *C. liquefaciens* (non-pathogenic human fungus) and *C. neoformans* (human pathogenic fungus) through analysis by high-resolution scanning electron microscopy (HRSEM), helium ion microscopy (HIM) and atomic force microscopy (AFM) and associates them with biological adaptation to the environment and to the host. A comparative analysis between the capsules of *C. neoformans* and *C. liquefaciens* demonstrated that both species produced branched capsules with different density areas which form a macromolecular network structure similar to a "microgel". These microgel are a result of multiple filament interactions. Furthermore, the structural similarities between both species generated similarities ($p>0.05$) in the interaction with *A. castellanii*, murine macrophages, NO production, secretion of a variety of immune mediators and in the survival of G. mellonella larvae infected by *C. neoformans* and *C. liquefaciens*. Finally, treatment with C. liquefaciens PS could not protect mice against infection with *C. neoformans.*

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Mechanisms of immunosuppression induced by cryptococcal capsular polysaccharide

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The prominent polysaccharide capsule from *Cryptococcus* spp is an essential virulence factor that has multiple effects on host immunity. This acidic and viscous polysaccharide, which is comprised mainly of glucuronoxylomannan (GXM), is continuously released by encapsulated yeasts during their replication. High levels of GXM in the body fluids of patients have a direct relationship on the severity of cryptococcosis. During different infectious diseases, as a crucial event of innate immunity, microbial molecules interactions with host phagocytic cells trigger inducible nitric oxide synthase (iNOS or NOS2) expression and nitric oxide (NO) release. This NO may have a dual role during infections, with both anti-microbial effector functions and immunosuppressive properties mediated by the apoptosis of inflammatory cells. Apoptosis is the most common and well-studied type of programmed cell death that helps to reduce the inflammatory stress, but it can also be manipulated by pathogens as a survival strategy. Two pro-apoptotic signal transduction pathways have been described: an extrinsic/receptor-linked apoptotic pathway and an intrinsic/mitochondria-mediated pathway, both activate downstream effector caspase-3. However, mitochondrial damage can also lead to a caspase-independent death through activation of death effectors, such as the apoptosis-inducing factor (AIF) and endonuclease G. In our laboratory, we have developed experimental models of cryptococcosis in rats, which have similarities with human infections showing tisular granulomas associated with inducible iNOS expression and NO production by macrophages. In this presentation, I will discuss experimental data describing mechanisms involved in macrophage apoptosis promoted by cryptococcal capsular polysaccharide (GXM) through NO generation. *In vitro* experiments using peritoneal macrophage cultured with purified GXM from *C. neoformans* demonstrated that GXM promotes iNOS expression with NO production in a dose-dependent manner. Likewise, capsular polysaccharide from *C. gattii* also elicited macrophage NO production, but to a lesser extent than GXM from *C. neoformans*. *In vivo*, intraperitoneal GXM administration in Wistar rats showed that most peritoneal cells were able to internalize polysaccharide and these cells produced elevated NO release. Furthermore, immunohistochemistry of lung tissue from GXM-treated rats also demonstrated that GXM induces iNOS expression *in vivo*.Experiments using blocking antibodies and flow cytometric or immunofluorescence analysis further revealed that NO production involves GXM binding by CD18 on macrophages. In contrast, GXM did not stimulate NO production via mannose or β-glucan receptors. Capsular polysaccharide also binds macrophages through Fcγ receptor II (FcRII), but this interaction provided inhibitory signaling for NO production. Engagement of CD18 on cellular surface induces activation of serine/threonine kinases, protein kinases C (PKC). According to this, our experiments show that PKC activation, but not tyrosine kinases (TK) or mitogen-activated protein kinases (MAPK), are involved in GXM-induced iNOS and NO production by rat macrophages. On the other hand, our data depicted that GXM-loaded macrophages undergo apoptosis after culture, and this phenomenon was fully prevented by the iNOS inhibitor, aminoguanidine (AG). Furthermore, the pathways involved in GXM-mediated NO production: CD18, FCγRII and PKC, also modulated in the same way the macrophage apoptosis. In agreement to the *in vitro* observations, polysaccharide-loaded tisular macrophages also presented apoptotic nuclei during disseminated cryptococcosis in rats. Strinkly, GXM-induced macrophage apoptosis was not prevented by the presence of a pan-caspase inhibitor (Z-VAD-fmk) in cultures, suggesting that apoptosis was independent of caspases activation. In contrast, GXM increased the mitochondrial membrane permeability and cytosolic AIF expression, showing that GXM triggers a caspase-independent cell death by promoting depolarization of mitochondria membrane potential. Taken together, these findings describe novel immunomodulatory mechanisms by the main cryptococcal capsular polysaccharide, which could contribute to limit inflammation during the infection

Zinc metabolism in *Cryptococcus*-host interface

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Zinc is a ubiquitous metal, as it is a structural component of the almost 10% of eukaryotic proteins. Due to its importance to all life forms, immune cells developed strategies to reduce zinc bioavailability to invading pathogens. Previous work from our group revealed that proper zinc homeostasis in *Cryptococcus gattiiis* important for virulence, as revealed in murine models of cryptococcosis. Furthermore, the defects in zinc uptake presented by *C. gattii* mutants deficient in zinc transportersled to reduced intracellular proliferation rate in macrophages. This suggests that macrophages expose engulfed cryptococcal cells to a low zinc environment. To gain information about this assumption, we evaluated the expression of zinc transporters of macrophage cells in the presence of *Cryptococcus neoformans*. In addition, the labile zinc levels were detected employing fluorescent probes. We found that cryptococcal cells led to a reduced intracellular labile pool of zinc in macrophages. This reduction was due to the alteration of zinc transporters expression, from both ZIP and ZnT families.Treatment of immune cells with additional extracellular zinc led to an increased outcome of cryptococcal cells. In order to assess the possibility of an evolutionary conserved mechanism of zinc restriction, we evaluated the zinc levels and the transcriptional response of zinc transporters in *Acanthamoebacastellanii* exposed to *C. gattii* WT and mutants deficient in zinc uptake. Exposure of *Acastellanni* cells to *C. gattii* also induces a drastic alteration of the zinc transporter transcript levels and a reduction of the intracellular zinc levels. Our results suggest that zinc restriction could be an evolutionary conserved mechanism to hamper cryptococcal development inside phagocytic cells.

Influenza virus as a predisposing factor for cryptococcosis

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Cryptococcus gattii is one of the main etiologic agents of cryptococcosis, a disease that affects lungs and the the central nervous system (CNS), causing meningoencephalitis in immunocompetent individuals. Considering the epidemiology of the disease, the predisposing factors for cryptococcosis caused by *C. gattii* are not well understood and it is possible that its pathophysiology may be influenced by other pathogens in a coinfection situation. The *influenza* A infections are a major public health concern and cause annual epidemics with significant morbidity and mortality worldwide. Considering that (i) both pathogens affect the respiratory tract and the (ii) relevance and severity of infections caused by *influenza* A and by *Cryptococcus* spp., we developed and studied an *in vivo* model of coinfection with *C. gattii* and *influenza* A and evaluated the influence of the virus in the progression of cryptococcosis. C57BL/6 mice were divided in the following groups: 1) infected with *influenza* A H1N1; 2) infected with *C. gattii* and 3) infected with *C. gattii* after viral inoculation. Mice infected with both pathogens have increased morbidity and early mortality compared to animals infected with only *C. gattii*; while animals only infected with the virus did not succumb. Furthermore, *influenza* A and *C. gattii* coinfection lead to a significant increase in the fungal load in the CNS and in the recruitment of neutrophils and lymphocytes to the bronchoalveolar lavage fluid. Macrophages previously infected with the virus have decreased ability to engulf and kill the fungus. The inefficient antifungal activity of macrophages was associated with the decreased levels of INF-γ (a key cytokine for the macrophage activation) in the lungs, which was a probable consequence of the increased expression of INF-α and INF-β in this organ. These results suggest that *influenza* A virus may be considered a predisposing factor for cryptococcosis. In addition,we provided new information about the interaction between these infectious agents and host during coinfection.

Role of calcium transporters on cryptococcal pathogenesis

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Cryptococcus neoformans have to cross the blood brain barrier (BBB)in order to access the central nervous system (CNS) and cause meningoencephalitis. There are three potential mechanisms by which yeast cell can cross BBB: transcellular, paracellular and by Trojan Horse. Hence,*C. neoformans* rely on different virulence factors to properly reach CNS. Enzymes as urease, phospholipase B and hyaluronic acid synthase are known as essential components to cross the BBB. The calcium (Ca2+) is a cellular second messenger that participates on calcineurin signaling, a pathway crucial and responsible for cryptococcal virulence and host adaptation. Moreover, the regulation of Ca2+ intracellular levels is coordinated by vacuolar Ca2+ transporters such as Pmc1 and Vcx1, which are the main subject of our study. Despite of many advances in understanding the molecular mechanisms of *C. neoformans* infection have been made, the molecular events that regulate dissemination to CNS are poorly understood. It has been shown that Vcx1 and Pmc1 are required for proper virulence of C. neoformans assessed in an intranasal model of murine cryptococcosis. Therefore the aim of this work wasto evaluate the impact of Vcx1 and Pmc1 on cryptococcal virulence and dissemination to the CNS. Murine infection was conducted by injection into the retro orbital space, which represents a systemic infection. While WT (H99 strain) and vcx1 null mutants were able to kill mice (LT50 of 9.5 and 7 days, respectively), *C. neoformans* cells lacking the PMC1 gene (pmc1 and pmc1/vcx1 null mutants) could not cause death in hosts, even after 30 days of infection. Fungal burden was accessed in lungs and brain 3 and 6 days post infection. The pmc1 and pmc1/vcx1 null mutants infectivity was shown to be highly impaired. Analysis of in vitro internalization and transmigration profiles was performed employing a monolayer of t-HUVEC cells using trans-well inserts to mimic the BBB. The null mutants pmc1 and pmc1/vcx1 showed impair ability to transmigrate.In order to understand the molecular mechanisms of such phenotypes,the global expression profile of pmc1 mutant strain was assessed through RNA-seq analysis to explore alterations on transmigration pathways. The disruption of PMC1 geneleads to alterations on the gene expression profile, mainly on pathways involved with paracellular mechanism of transmigration,calcium homeostasis and calcineurin signaling pathway.Collecting these results, we intend to determine the role of calcium transporters on *C. neoformans* virulence, and their role upon virulence factors that contributes for transmigration to the CNS

Prevalence of Cryptococcal Antigenemia in Latin America

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Cryptococcal meningitis represents the main cause of HIV-related opportunistic meningitis in Latin America and mortality continues to be unacceptably high. Early diagnosis of cryptococcal infection is a keystone to improving outcomes. Detectable cryptococcal antigen (CRAG) in peripheral blood precedes symptomatic meningitis disease by weeks to months, offering an opportunity for early detection and preemptive intervention. The World Health Organization (WHO) recommends routine serum or plasma CRAG screening in antiretroviral therapy-naïve adults with CD4 counts <100 cells/μL, followed by preemptive anti-fungal therapy if CRAG positive, to prevent the development of cryptococcal disease. WHO recommended CRAG screening among populations with a prevalence of cryptococcal antigenemia of >3%. Thus, in order to implement WHO recommendations, it is necessary to know the local CRAG prevalence. Nowadays, few studies about prevalence of asymptomatic cryptococcal antigenemia were performed in several subsets of patients in Latin America. The reported prevalence including inpatients and outpatients ranged from 2.7% to 6.2%. These results suggest that routine CRAG screening may be beneficial in our region. However, CRAG screening implementation presents individual and public health challenges. On the other hand, whole blood CRAG lateral flow assay screening seems to be a simple strategy in this scenario.

Cryptococcosis mortality and Aids in Brazil (2000 to 2012)

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Cryptococcosis is a neglected and predominantly opportunistic cosmopolitan mycosis, highlighted as the most frequent among the systemic mycoses associated with aids (PRADO, et al, 2009) and an important public health problem in Brazil, characterized by late diagnosis and high lethality (up to 65%). The present study analysed cryptococcosis mortality and aids in Brazil from 2000 to 2012 based on secondary data (Mortality Information System/SIM-DATASUS and IBGE). Of a total of 5,755 recorded deaths in which cryptococcosis was mentioned as one of the morbid states that contributed to death, two distinct groups are discernible: 1,121 (19.5%) registered cryptococcosis as the basic cause of death and 4,634 (80.5%) cryptococcosis associated with risk factors, mainly aids (75%), followed by other host risks (5,5%). The mortality rate by cryptococcosis as the basic cause was 0.47/million inhabitants, whereas the mortality rate by cryptococcosis as an associated cause was 1.94/million inhabitants, resulting in a total rate of 2.41/million inhabitants when all mentioned cases were considered. Meningitis was the predominant clinical form (80%), males were the more affected (69%), and 39.5 years old was the mean age. The highest mortality rates due to cryptococcosis as basic cause occurred in the states of Mato Grosso, (0.84/million inhabitants, Central-West and Pará, (0.82/million inhabitants, the North region. Mortality rates due to cryptococcosis as associated cause were higher in the states of Santa Catarina (5.42/million inhabitants) and Rio Grande do Sul (4.95/million inhabitants), both in the South Region.The observed trends for mortality rates were upward in the Northeast ($p = 0.001$) and downward in the Southeast ($p < 0.001$) regions, both statistically significant. It is worth mentioning that the mortality rates by meningitis due to virus, tuberculosis and toxoplasmosis are lower than the mortality rate by cryptococcal meningitis (0.37/millions inhabitants), only surpassed by meningococcal meningitis (0.76/millions inhabitants), despite the fact that 77% of the meningitis cases were with no defined etiological agent. This study is relevant because it shows the magnitude of cryptococcosis mortality in aids and can contribute to control and surveillance programs and highlight attention to the urgent prioritization of early diagnosis and proper treatment to reduce the unacceptable lethality rate of this neglected mycosis in Brazil.

Diagnosis of cryptococcal disease: before and after the IMMY CrAg LFA

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Cryptococcal meningitis (CM) is the most common cause of meningitis in adults in Africa, accounting for over 500,000 deaths annually. In the USA,CM is more common than all causes of bacterial meningitis combined, at an incidence of 1.1 per 100,000 versus 0.728 per 100,000, respectively.Cryptococcal antigen in blood can be detected a median of 22 day before symptoms of meningitis develop, thus enabling the identification of patients who could receive pre-emptive therapy. Pre-emptive therapy significantly increases survival times. In 2011, the World Health Organization recommended routine screening for cryptococcal antigen in low CD4 count HIV patients, and subsequently 22 countries have incorporated similar screening strategies into their countries' guidelines. Currently, no Latin American counties have recommendations for routine cryptococcal antigen screening despite published CrAg prevalence rates of 3-6% in Argentina, Brazil, Colombia, Guatemala, and Peru.The IMMY Cryptococcal Antigen Lateral Flow Assay (CrAg LFA) was released in 2011, and quickly became the gold standard for diagnosis of cryptococcal disease. The CrAg LFA meets the WHO A.S.S.U.R.E.D criteria with an easy 5-step procedure, a time-to-result of 10 minutes, sensitivity and specificity of ~99%, and no cold-chain requirement. The CrAg LFA is approved for use with venous & capillary whole blood, serum, plasma and CSF (Ex-USA) and is approved for use with serum and CSF (within USA). The CrAg LFA has many advantages over CrAg latex or CrAg EIA for implementation of CrAg screening programs.Early diagnosis using the IMMY CrAg LFA with pre-emptive treatment is saving thousands of lives every year by preventing the development of CM. The time is now for Latin American countries to implement similar life-saving strategies.

Lateral flow assay in the early diagnosis of cryptococcosis in severely immunosupressed AIDS-patients from the Midwest Region of Brazil

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The predominance of cryptococcosis (CRC) in AIDS-patients severely immunosupressed, its high lethality rate, and the short survival time despite appropriate treatment suggest that the early diagnosis is the best approach to face this coinfection. A study was performed with 188 AIDS-patients from the Midwest Brazilian Region with severe immunosupression, characterized by TCD4$^+$ lymphocytes count of 86.5±60.3/mm^3 and no previous diagnosis of CRC. These patients were 41.5±11.3 years old and the diagnosis of AIDS lasted 4.5±4.6 years; they showed a 13.9% positivity of antigenemia by the lateral flow assay – LFA. The comparison of the neurological manifestations in the groups comprising patients LFA+ and LFA- showed no differences, demonstrating that central nervous system findings could not be used as a clinical marker for further laboratory investigation. Thus, an active search should be performed for an early diagnosis. The comparison of four tests showed higher positivity of LFA (13.9%) and latex agglutination test – LAT (11.3%), with no difference between them ($p>0.05$). Blood and urine culture (BC=4.4% and UC=3.9%; $p=0.56$) showed lower positivity than both serological assays [(LFA=LTA) > (BC=UC); $p<0.05$]. The parameters of accuracy of LFA for cryptococcosis were sensitivity of 87.5%, specificity and positive predictive value of 100%, negative predictive value of 98.0%, accuracy of 98.3%, positive likelyhood ratio of 172.3, and negative likelyhood ratio of 0.15. Cross reactions with some systemic mycoses were rare – histoplasmosis (5.3%) and paracoccidioidomycosis (5.3%), or absent (aspergillosis). The high incidence of positive results in this region and the severe progress of CRC in AIDS-patients strongly suggest an active search of this coinfection. Based on our results, the antigen detection should be the method of choice. In addition, LFA presents good accuracy, and is easier to perform and faster to provide results few minutes after blood collection, during the appointment, permitting an immediate medical decision on treatment.

New approaches into antifungal susceptibility testing for *Cryptococcus* isolates

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The minimum inhibitory concentration (MIC) that is traditionally used to guide therapy is, up to now, worthless in the managements of cryptococcosis cases. New tests indicate the occurrence of subpopulations heteroresistant fluconazole and/or tolerant to amphotericin B among clinical strains of *Cryptococcus neoformans* and *C. gattii*. The mechanisms of AMB-t or FLC-h are still not totally elucidated, and to date, no studies addressing the clinical relevance of these forms of resistance was reported. Such resistance in environmental isolates of *Cryptococcus* species agents may also occur, although its extent is unknown. Both forms of resistance may constitute tools for future prognosis of Cryptococcosis cases treated with amphotericin B and/or fluconazole.

Susceptibility testing and epidemiological cut off. How to use in clinical practice?

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INEI "Dr C.G. Malbrán"

The *Cryptococcus neoformans* species complex (*C. neoformans*) causes severe infections in patients with and without immunocompromised condition, being HIV/AIDS patients the most susceptible population. The management and treatment of cryptococcal meningitis is difficult due to the high frequency of relapse episodes. A high rate of mortality reaches up to 35% during the first months after diagnosis and survival is estimated as 1-2 years post-diagnosis. The recommended treatment is amphotericin B with or without flucytosine followed by fluconazole at the consolidation period. However, the response to conventional therapy is unpredictable; recurrence is common, treatment failure reaches 20-30%, and, in most cases, the patient dies.The role of antifungal susceptibility testing is to aid in selecting the best antifungal drug for treatment. However, the available reference methods to determine the *in vitro* susceptibility against antifungal drugs, the M27-A3 and EDef 7.3 of the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST), respectively, do not include breakpoints for any antifungal drug against *C. neoformans* isolates. Thus, susceptible/resistant categories should not be used due to the absence of interpretative breakpoints for this yeast. This is a critical point due to the difficulties in interpreting the *in vitro* antifungal susceptibility results. Epidemiological cutoff values (ECVs) based on the distribution of minimal inhibitory concentrations (MICs) have been recently proposed for some antifungal drugs against genotyped and non-genotyped strains of *C. neoformans*. The ECV is a useful tool to distinguish the wild-type (WT) population, which is the population of microorganisms in a microorganism/drug combination with no detectable acquired resistance mechanism, from the non- wild type (non-WT) populations, which are those that may exhibit acquired or mutational resistance mechanisms to the drug in question. On the other hand, species-specific clinical breakpoints (CBP) categorize the isolate as treatable (susceptible) or non-treatable (resistant), and is the most accurate predictor of patient response to treatment. CBP determination is based on MIC distributions, PK/PD parameters, animal models, clinical outcome and therapy, and could provide data more useful for guiding therapy. To date, CBPs are not available for *C. neoformans*. In absence of CBPs the common question is if the ECV should be useful to guide the clinical treatment. Opinions vary. The ECVs may aid in identifying the circulating strains with decreased susceptibility to the antifungal drugs evaluated. Also, the values obtained might be useful for early detection of decreased susceptibility of a population of microorganisms. However, these values should not be used to determine clinical resistance. To remark, the CLSI are considering reporting ECVs where CBPs are not available, including a paragraph that indicates whether the isolate is WT or non-WT, without categorical interpretation of susceptible/resistant. In conclusion, ECVs may be clinically useful in detecting non-WT isolates that have reduced susceptibility against antifungal drugs.

Cryptococcosis and its agents in Amazonia

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Cryptococcus neoformans and *Cryptococcus gattii* are responsible globally for almost one million cryptococcosis cases yearly, mostly in immunocompromised patients, such as those living with HIV. Its agents (*C. neoformans* and *C. gattii*) present eight major molecular types—VNI-VNIV and VGI-VGIV respectively. Phylogenetic and recombination analyses, based on fingerprint and multiple MLST datasets with Amazon isolates have been demonstrating that the main agents are *C. neoformans* (VNI) and *C. gatti* (VNII) and MLST analysis presented important St's in Amazon Region. The aim of this lecture is discuss about Cryptococcosis and its agents in Amazonia, including environmental and clinical isolates.

On the origin and dispersal of *Cryptococcus neoformans* var. *grubii*

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Cryptococcus neoformans var. *grubii*(*Cng*) can broadly be divided into three lineages: VNB, VNI and VNII. Previous population genetics approaches have raised the 'Out of Africa' hypothesis to account for the proliferation of this lineage worldwide. However, the investigation into the likely geographical origin still needs investigation. We used whole genome sequencing (WGS) of isolates sampled worldwide to infer the population genetic structure of *Cng*, to subsequently build a scenario for the evolution of this lineage. A collection of 165 diverse isolates have been sequenced from across the globe, with a focus on Latin America, Africa, the Indian subcontinent and South-East Asia. Analysing population structure whilst accounting for linkage has revealed high levels of sharing of genetic material within lineages, with evidence for ancestral recombination events for all three lineages originating in Latin America. These analyses have also revealed similar events in Africa and India in the VNI lineage, presenting hypotheses for the diversification and spread of these lineages across the globe. Phylogenomic analyses have also revealed a migration of the VNB lineage between African and Latin America during diversification, indicating that more than a single exchange of VNB has occurred between these continents.

Epidemiology of *C. neoformans* var. *neoformans*

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Cryptococcus neoformans is distinct in two varieties: *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans.* The two varieties are morphologically indistinguishable but they can be identified biochemically on creatinine dextrose bromothymol blue thymine (CDBT) medium. Also molecular typing is able to distinguish the two varieties identifying three molecular types for var. *grubii* (VNI, VNII, and VNB) and one for var. *neoformans* (VNIV). VNI molecular type causes most of the cases of cryptococcosis worldwide, in contrast VNIV is less frequent. VNIV isolates have been reported mainly in Europe (18%) and Northern America (6%), but they are also present in South America, Asia, Oceania, and Africa (1-2%). Interestingly, Europe and North America present the highest prevalence of intervarietal hybrids confirming the important role played by var. *neoformans* in the hybridization process. Both mating type alpha and a of this variety have been isolated from clinical and environmental sources with a ratio, in Europe, of about 6:1, respectively. However, the frequency of MATa isolates in the environment was not homogeneous presenting a hotspot in Greece. Clinical manifestations of infections due to *C. neoformans* var. *neoformans* are similar to those observed in var. *grubii* infections with primary involvement of the lungs and then dissemination to brain. Cutaneous manifestations are reported to be associated to this variety being prevalently isolated from skin lesions due to both primary and secondary cutaneous cryptococcosis. Isolates from veterinary sources have been also isolated from cats, dogs, cows, squirrels, goats, and mouflons. In the environment it has been recovered from bird excreta, dust, soil, and arboreal material. Recently, we showed that var. *neoformans* is able to grow at low temperature (10°C) better than var. *grubii* allowing to the former to survive in environments characterized by a cold season. In addition, we showed that it is more resistant to sunlight exposition than var. *grubii* and therefore it has a higher fitness for niches like bird excreta and tree bark which offer a low protection from sunrays. Genetic population studies on a large number of *C. neoformans* var. *neoformans* isolates from different geographical origin and from different sources support the hypothesis that this population is recombinant. The high haplotype diversity as well as the low evolutionary divergence among *C. neoformans* var. *neoformans* population confirm that isolates are strictly correlated each to the other but they are characterized by high variability due to recombination. Statistical tests such as linkage disequilibrium analysis, Watterson estimator calculation, and recombining events evaluation also corroborate this hypothesis. In addition, a recent genetic structure study carried out in Europe showed that *C. neoformans* var. *grubii* clinical isolates are more correlated to an arboreal origin than var. *neoformans*. Although some studies have produced new important results about *C. neoformans* var. *neoformans*, many questions remain to be explored in future to understand the epidemiology, ecology and biology of this pathogen

Molecular Epidemiology of *C. gattii* VGII – Brazil and its global connection

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Cryptococcosis is an emerging human fungal infection of significant mortality and morbidity in Latin America, especially in the form of meningitis. The case-fatality rate are unacceptably high - vary from 42.7% to 49.6% in Brazil. Despite this, it is not included in the list of neglected diseases and is not notified. Thus, the incidence and prevalence of cryptococcosis are little known. Infections due to *Cryptococcus gattii* have mainly been described in tropical and subtropical regions, but its adaptation to temperate regions was crucial in the species evolution and highlighted the importance of this pathogenic yeast in the context of disease. *C. gattii* molecular type VGII has come to the forefront in connection with an on-going emergence in the Pacific North West of North America. Taking into account that previous work pointed towards South America as an origin of this species, the present work aimed to assess the genetic diversity within the Brazilian *C. gattii* VGII population in order to gain new insights into its origin and global dispersal from the South American continent using the ISHAM consensus MLST typing scheme. The results corroborate the finding that the Brazilian *C. gattii* VGII population is highly diverse, and the diversity is likely due to recombination generated from sexual reproduction, as evidenced by the presence of both mating types in clinical and environmental samples. The high genetic variability of Brazilian VGII strains has been inferred using 145 strains from 4 out of 5 Brazilian regions. A total of 81 sequence types and haplotype diversity (Hd) equal to 0.978were observed, revealing a high genetic variability among the VGII Brazilian strains. Besides, the numerous genotypes represent a link between Brazil and other parts of the world reinforcing South America as the most likely origin of the *C. gattii* VGII subtypes and their subsequent global spread, including their dispersal into North America, where they caused a major emergence. Furthermore, the isolates from the transitional ecological area in Northeast Brazil are the most likely ancestor lineages, translocating from caatinga/cerrado by adapting progressively throughout Amazonia, and then, spread to the North American Pacific Northwest regions and other parts of the world on multiple occasions. This picture is intrinsically related to climatic changes and devastating human activities globally. Therefore, a multifocalorigin for the outbreak lineages of cryptococcal infections must be considered.

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Novel insights in the molecular epidemiology of *Cryptococcus gattii* VGIII

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From the four major molecular types (VGI-VGIV), in which *Cryptococcus gattii* is traditionally classified, the molecular type VGIII has recently emerged as cause of disease in otherwise healthy individuals and animals. This rising incidence has brought about the need to investigate the population genetic structure of this molecular type, in order to understand if there are potential genotype-dependent characteristics in its epidemiology, environmental niche(s), host range and clinical features. Thanks to the global cooperation that exists between research groups devoted to the study on *Cryptococcus* and cryptococcosis, a representative set of clinical, environmental and veterinary *C. gattii* VGIII isolates from endemic areas and sporadic cryptococcosis cases around the world, have been collected and characterized by genotypic and phenotypic methods. Both multilocus sequence typing (MLST) and whole genome sequencing (WGS) showed that the *C. gattii* VGIII population is genetically highly diverse, with minor differences between countries, isolation source, serotype and mating type. The isolates grouped into four well-supported diverging sub-populations, with the two main clades, containing the majority of the isolates, corresponding to serotype B or C, respectively, indicating an ongoing species evolution, which is supported by little to no recombination between these two major groups, not only at the whole genome level but also and the mitochondrial level. Importantly, both major clades included clinical, environmental and veterinary isolates. Phylogenetic analysis also revealed two more distant, but basal groups in the VGIII population. WGS revealed that Mexico and USA are likely the origin of the serotype B VGIII population while Colombia is the possible origin of the serotype C VGIII population, which is in accordance with the fact that *C. gattii* VGIII is widespread in the Americas, with sporadic cases occurring elsewhere. Phenotypically, serotype B isolates showed to be more virulent than serotype C isolates in a murine model of infection, causing predominantly pulmonary cryptococcosis, although no specific link between genotype and virulence has been observed. Antifungal susceptibility testing against six antifungal drugs revealed that serotype B isolates are more susceptible to azoles, the primary treatment for uncomplicated cryptococcosis, than serotype C isolates, highlighting the importance of strain typing to guide effective treatment to improve the disease outcome, and reduce morbidity and mortality in both sporadic cases and those associated with outbreaks. These findings have significantly contributed to the understanding of the epidemiology, genetics and pathogenesis of the *C. gattii* VGIII population.

Tamoxifen-boosted antifungal therapy for cryptococcal meningitis – trial rationale and design

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In Vietnamese patients with cryptococcal meningitis the death rate at 10 weeks and 6 months is 31% and 35% for patients receiving gold standard treatment with amphotericin and flucytosine.This compares with 33% and 46% in patients receiving amphotericin-fluconazole induction therapy and 44% and 54% in patients receiving prolonged amphotericin monotherapy. In practice, the rate of clearance of yeast from cerebrospinal fluid (CSF) is slow, with only around 50% of patients having sterile CSF after one week of treatment. Randomised controlled trials from Vietnam and elsewhere suggest that greater rates of clearance are associated with better clinical outcomes. Tamoxifen is an off-patent, selective estrogen receptor modulator (SERM) used most frequently for treatment of breast cancer, but also other tumours, including primary brain tumours. It has been demonstrated to have activity against *C. neoformans* in vitro having synergy when combined with amphotericin. Moreover, in the mouse model of cryptococcosis it appears to have a fungicidal effect when combined with fluconazole. It is concentrated in lipid rich tissues including the brain, and in the phagosomes of macrophages. It is therefore a candidate drug to improve outcomes from cryptococcal meningitis. In this short talk will present susceptibility data of Vietnamese clinical strains to tamoxifen including when in combination with other antifungal drugs, and our design for a randomised controlled trial of tamoxifen boosted antifungal therapy, illustrating some of the challenges around this potential widely available and affordable treatment.

Challenges of a paradoxical inflammatory syndrome in non-HIV cryptococcosis

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In HIV-related cryptococcal disease, defects in T-cell immunity are paramount, whereas there is little understanding of mechanisms of susceptibility in non-HIV related disease, especially that occurring in previously healthy adults. In our work toward developing treatment options, detailed immunological studies of those with severe central nervous system (s-CNS) disease defined as those refractory to standard anti-fungal therapy were conducted to 1) identify mechanisms of susceptibility as well as 2) understand mechanisms underlying severe disease. Despite the expectation that, as in HIV, T-cell immunity would be deficient in such patients, cerebrospinal fluid (CSF) immunophenotyping, T-cell activation studies, soluble cytokine mapping and tissue cellular phenotyping demonstrated that patients with s-CNS disease displayed strong intrathecal expansion and activation of cells of both the innate and adaptive immunity including HLA-DR+ CD4+ and CD8+ cells and NK cells. These expanded CSF T cells were enriched for cryptococcal-antigen specific CD4+ cells and expressed high levels of IFNG as well as a lack of elevated CSF levels of typical T-cell specific Th2 cytokines -- IL-4 and IL-13. However, while tissue macrophage recruitment to the site of infection was intact, polarization studies of brain biopsy and autopsy specimens demonstrated an M2 macrophage polarization and poor phagocytosis of fungal cells. These studies expand the paradigm for cryptococcal disease susceptibility to include a prominent role for macrophage activation defects and suggest that severe neurological disease is characterized by a paradoxically elevated, but ineffective T cell responses.

The AMBITION Study: high dose short course liposomal amphotericin for HIV- associated cryptococcal meningitis

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Early mortality in HIV programmes in Africa is considerably higher than in high-income countries. Almost 20% of these deaths are directly attributable to cryptococcal meningitis (CM). Current treatments are inadequate. Ten- week mortality is ~60% with fluconazole treatment, the current standard-of-care in most of Africa. Conventional 2-week amphotericin B induction is more effective, but not widely available outside South Africa; use is limited by serious toxicity and the need for intensive nursing care and laboratory monitoring. New treatments are urgently needed. Recent data suggest highly effective and much safer therapy for HIV-associated CM is possible with a novel short-course of high-dose liposomal amphotericin (L-AmB, Ambisome), a newer formulation of amphotericin B. We have recently completed a phase-II study showing that a single 10mg/kg dose of L-AmB is safe, and leads to rapid clearance of infection. We are now commencing a multi-centre phase-III randomised non-inferiority trial to determine whether short-course high-dose L-AmB is as effective as 14-day amphotericin B-based therapy in averting all-cause mortality in HIV-associated CM. Treatment arms (both given with fluconazole 1200mg/d) will be: -Amphotericin B deoxycholate 1.0 mg/kg/day for 14 days (control) -L-AmB 10 mg/kg day 1 (single dose), which was the shortest-course L-AmB regimen tested in our recent phase-II EFA study that demonstrated an acceptable efficacy and safety profile.850 patients will be recruited at 6 African partner-sites, making this the largest HIV-associated CM trial ever conducted: University of Cape Town, Botswana-Harvard Partnership, University of Zimbabwe, Malawi-Liverpool- Wellcome Trust Clinical Research Unit, UNC Project Malawi, and National Institute of Medical Research, Tanzania. The primary European partners are LSHTM and the Institut Pasteur, plus St. George's University of London, Liverpool University and the Liverpool School of Tropical Medicine. The partnership brings together leading European and African HIV and cryptococcal research groups. A novel short-course highly effective and safer L-AmB treatment regimen for CM would transform the management of late-stage HIV and markedly improve outcomes in HIV programmes in Africa.

ACTA (Advancing Cryptococcal meningitis Treatment for Africa) Update

Síle Molloy
ACTA Study Team

A phase III, randomized, controlled non-inferiority trial for the treatment of HIV-associated cryptococcal meningitis: Oral fluconazole plus flucytosine or one week amphotericin B-based therapy vs two weeks amphotericin B-based therapy. Cryptococcal meningitis (CM) is a leading cause of death among HIV-infected people in resource-poor settings. In excess of 100,000 deaths are attributable to CM annually in sub-Saharan Africa, with mortality in routine care estimated at 70% at 10 weeks. A 14-day course of amphotericin-B deoxycholate (AmB) with flucytosine is the recommended treatment. However, AmB requires intravenous administration and intensive monitoring to manage toxicities, and flucytosine is unavailable in most resource poor settings. The widely-used alternative, high dose oral fluconazole monotherapy, is inadequate at clearing the infection, thus necessitating new, sustainable treatment regimens for this region. The trial compares an optimized oral combination regimen, and a short course AmB-based regimen, with the recommended 14-day AmB combination therapy for efficacy for initial treatment of CM. Fluconazole and flucytosine will also be compared as the second agent when combined with AmB. A cost-effectiveness study is being undertaken alongside the trial.An open label, phase III, randomized-controlled, non-inferiority trial is being conducted in 9 sites in Malawi, Zambia, Cameroon and Tanzania to compare 3 strategies for CM treatment in 680 HIV-seropositive adults (≥18 years old) with a first episode of CM. The trial aims to determine whether alternative induction regimens of; 1. Fluconazole (1200mg/day) plus flucytosine (100mg/kg/day) given orally for 2 weeks; or 2. AmB (1mg/kg/day) plus fluconazole (1200mg/day), or flucytosine (100mg/kg/day), for 7 days, are non-inferior to 3. AmB (1mg/kg/day) plus fluconazole (1200mg/day), or flucytosine (100mg/kg/day), for 14 days. After 2 weeks, all participants receive consolidation and maintenance fluconazole. ART is initiated at 4 weeks in accordance with national protocols. The primary endpoint is all-cause mortality at 2 weeks. Secondary endpoints include all-cause mortality at 4 and 10 weeks, rates of clearance of infection, and tolerability of the regimens. In addition, micro-costing of the treatment regimens is undertaken in Zambia. The identification, through this trial, of a sustainable and effective treatment regimen for CM could lead to a substantial reduction in mortality among HIV-infected patients in resource poor settings.

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Fungal Phosphate Uptake at host pH- a passport to the CNS

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During a host infection, *Cryptococcus neoformans* experiences different microenvironments which impact its ability to derive nutrients. In lung and brain, *C. neoformans* grows as dense masses(cryptococcomas), which provide a more acidic microenvironment than the blood.Within its various host niches, *C. neoformans* must obtain an adequate supply of essential nutrients, including phosphate, and tolerate host stress to achieve a successful infection.The acquisition of phosphate by fungi is regulated by the PHO signaling cascade, which is activated when intracellular phosphate falls below a critical level.PHO pathway activation leads to induction of transporters,which allow uptake of free phosphate.By blocking the PHO pathwayusing a Pho4 transcription factor mutant (*pho4*Δ), we demonstrate theimportance of this pathway for dissemination of *C. neoformans*to the CNS and the establishment of meningitis in murine models. We also found that *pho4*Δwas more susceptible than WT to a number of stresses, including nitrosative stress and alkaline pH.Except for alkaline pH, allstresssusceptibilities were alleviated by phosphate supplementation. We found that the near-abolition of*pho4*Δdissemination from lungs to the CNS is partly due to its inefficiencyto take up phosphate at alkaline pH. Cryptococcal growth at alkaline pH therefore mimics the condition of phosphate starvation, requiring PHO pathwayupregulation to replenish intracellular phosphate. Given that *pho4*Δ cannot upregulate its PHO pathway, the mutant experiences inhibitedproliferationand hypersusceptibility to host-immune stress,particularly in the blood.

A plea for recognizing cryptic species in the *Cryptococcus neoformans* / *Cryptococcus gattii* complex

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Cryptococcosis is an important fungal infection globally affecting both immunecompromised and immunocompetent human beings and caused by members of the *C. neoformans* / *C. gattii* complex. It has been estimated that annually nearly a million HIV-positive individuals develop cryptococcal meningitis with > 600,000 casualties. One of the first sign of phenotypic heterogeneity within *C. neoformans* was based on the antigenic properties of the polysaccharide capsules. The sexual cycle of Cryptococcus was discovered in the 1970's which led to the description of Filobasidiellaneoformans for *C. neoformans* var. *neoformans* and Filobasidiellabacillispora for *C. neoformans* var. *gattii*. In a recent revision of the taxonomy of basidiomycetous yeasts the name Cryptococcus was given priority over Filobasidiella and, moreover, this name was limited to the so-called Filobasidiella-clade. Prior to the latest proposed taxonomic revision of C. neoformans, two varieties were discerned as *C. neoformans* var. *neoformans* (serotype D) and *C. neoformans* var. *grubii* (serotype A). However, serotype AD hybrids are known for a long time and in several parts of the world form a major part of the infectious agents. From a nomenclatural point of view it is noteworthy that the type strain of *C. neoformans*, CBS 132, is a serotype AD hybrid and, thus has a mixed genetic background. In the late 1960's an atypical clinical cryptococcal isolate was obtained and named *C. neoformans* var. *gattii*. In 2002, *C. neoformans* var. *gattii* was raised to the species level as *C. gattii* representing isolates with serotypes B and C. However, with the advent of molecular techniques it became obvious that both *C. neoformans* and *C.gattii* were genetically more diverse than previously assumed. With the increasing resolution of molecular epidemiological tools, such as PCR-fingerprinting, amplified fragment length polymorphism (AFL) fingerprinting, multi-locus sequence typing (MLST), and whole genome sequencing (WGS), it was repetitively observed that irrespective of the application of these techniques the cryptococcal isolates fell in identical clusters. These insights were elaborated, presented and discussed at various ICCC meetings, starting with ICCC4 (London 1999) until ICCC9 (Amsterdam 2014). At ICCC6 (Boston 2005) a dedicated debate was held entitled '*Cryptococcus neoformans*: One, two or more species'. During this debate mainly two diverging opinions were presented and defended, namely a two-species concept and a multiple (at that time a six-species) species concept. During this debate the audience was asked to comment on the use of various names of the various clades and a large part of the audience supported the name *C. neoformans* for serotype A isolates. The type strain of *C. nasalis* belongs to serotype D and, hence, had nomenclatural priority over any other name. However, the audience of ICCC6 was against use of the name *C. nasalis* and preferred other names. Hence *C. deneorofmans* was proposed for this clade as it: 1. indicated affinity with the epithet *neoformans*, and 2. it also refered to serotype D (*de-neoformans*). The name *C. gattii* got renewed attention as it was reported as the cause of a number of major outbreaks which has led to an increase in the number of publications using that name. It is rather unfortunate that the rules of fungal nomenclature do not allow that this name being used for another clade than where the type strain belongs to (remember that the name *gattii* has been actively used in many other publications as well). Thus the clade usually referred to as VG1/AFLP4 represents *C. gattii*. Similarly, *C. bacillisporus* refers to the clade usually referred to as VGIII/AFLP5 as the type strain belongs here. Three other consistently observed clades in the *C. gattii* complex were named using '*gattii*' in part of the epithet in order to keep reference to the name '*gattii*'. To further complicate the taxonomy of the species complex, various hybrids have been described between members of the *gattii* complex and the *neoformans* complex. However, this level of genetic complexity is also seen in other yeast genera, such as Saccharomyces, where 'genetically clean' species occur that form hybrids and even triple hybrids. In the 2015 taxonomic revision of the *C. neoformans/C. gattii* species complex information is provided that isolates belonging to the various [sibling/cryptic] species differ phenotypically and that they can be identified by e.g. MALDI-TOF MS. In the anticipated presentation we will further present and discuss the above mentioned arguments and phenotypic differences between the species, including susceptibility values to antifungals.

Nomenclature from the global population genetic perspective

Wieland Meyer and the ISHAM working group on Genotyping *Cryptococcus neoformans* and *C.gattii*
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The taxonomy/nomenclature of the etiological agent(s) of cryptococcosis has been chaotic in the early years, till it became stabilized by 1950 as a single species *Cryptococcus neoformans*. Studies of the life cycle ecology, biochemistry, epidemiology and genetics revealed that two rather than one species are causing human/animal cryptococcosis, *C. neoformans* and *C. gattii*. Advancements in molecular biology have enabled a more detailed investigation of those two species. Using DNA- and PCR-fingerprinting early on, followed by *URA5*- or *PLB1*-RFLP analysis, AFLP, MLMT, MLST and more recently whole genome sequencing (WGS) analysis resulted in the detection of multiple genetically diverse monophyletic clades within each of the two species. In 2009 a consensus MLST typing scheme was adopted by the wider *Cryptococcus* research community, establishing a unified nomenclature of the different major molecular types, as VNI-VNIV for *C. neoformans* and VGI-VGIV for *C. gattii.* In addition a large number of intra- and interspecies or hybrids, with the most common one being the AD hybrid, have been identified. To improve the amplification efficiency new primer sets for all 7 MLST loci of the 2009 published consensus scheme have been developed, enabling now for an amplification of all loci from all *C. neoformans* and *C. gattii* strains at the same amplification conditions. In 2015 a proposal based 115 strains was published lamping *C. neoformans* into 2 species, and splitting *C. gattii* into five. However, population genetic analysis based on the published MLST data of 2,606 strains shows a greater genetic diversity as discovered by those 7 proposed species. On one side, the analysis of 1521 published *C. neoformans* strains has revealed 4 major haploid molecular types, represented by 210 MLST sequence types. These include the closer related molecular types VNI (AFLP1), VNB (AFLP1A) and VNII (AFLP1B) within *C. neoformans* var. *grubii* (serotype A) and the distant major molecular type VNIV within in *C. neoformans* var. *neoformans* (serotype D). In addition there are 10 genetic groups, which are placed distinctly from those major molecular types, these genetic groups share genetic information from the major molecular groups within *C. neoformans* and maybe the result of ancient or recent recombination. Further studies are needed. These findings do not support the lumping into only two species within *C. neoformans.*On the other side, the analysis of 1085 published *C. gattii* strains has revealed also four major haploid molecular types represented by 299 sequence types. These include VGI (AFLP4), VGII (AFLP6), VGIII (AFLP5) and VGIV (AFLP7) all encompassing isolates of both serotypes B and C. Further detailed genetic analysis, including WGS has even further divided the major molecular type VGIII into tow none combining major groups, corresponding either to serotype B or C. Overall these results support a separation of et least 4 species within *C. gattii.* The newly, based on two isolates from a single isolation event, described species "*C. decagattii*", characterised by isolates, which are by MLST analysis show a genetic profile typical for VGIII, but are by *URA5*-RFLP analysis belonging to the major molecular type VGIV, due to a mutation at the restriction site used for the RFLP analysis, can not be supported. DNA barcoding gap analysis, including only the two currently recognised "*C. decagattii*" isolates and the VGIII isolates indicated a DNA barcoding gap. However, when all between VGII and VGIV intermittent isolates are included in the DNA barcoding gap analysis there is no barcoding gap revealed between "*C. decagattii*" and the actual VGIII isolates. WGS analysis has confirmed the four major groups in *C. neoformans* as well as in *C. gattii*. As is obvious from the current data, the addition of further isolates will most certainly increase the number of genetic difference found or blur current species borders, and as such, individual naming of each of those clades at this point in time may inadvertently cause nomenclature instability and taxonomic confusion in a clinical setting. For that reason it is currently recommended to continue with the use of the *C. neoformans and C. gattii* species complex, in combination with the determination of the major molecular types, till conclusive studies, revealing molecular, biochemical, clinical and epidemiological data, have been conducted.

A phylogenomic view of the *Cryptococcus* species complexes

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Fungal speciation continues to be problematic due to a number of issues related to reproductive, morphologic, ecologic, and genomic ambiguities. While morphotyping, phenotyping and genotyping have been used to design at species and subtypesand infer relationships among fungal isolates (which are typically the result of clinical, veterinary or environmental convenience sampling), these methods are limited in their abilities to establish true genetic relatedness or establish population structure. Advances in whole genome sequencing and bioinformatics analyses now allow for us to establish current and historical relationships among proposed population groups and model their evolutionary relationships. Here we analyze the genomes of representatives of the major accepted subtypes of the pathogenic *Cryptococcus* species complexes, namely the *C. neoformans* complex and the *C. gattii* complex. Phylogenomics clearly identifies their previously established VN and VG subtypes, respectively. Newer subtypes, such as the reported bifurcation of VNI into two distinct clades, and of VGIII into multiple lineages, are also phylogenetically clear. What is not clear is the mutational differentiation threshold that distinguishes subtypes and species. Additionally, distinct lineages can be represented by individual strains, which in turn may also represent new molecular types/species.The placement of such “clades” within the phylogeny of a species complex, however, may represent serendipitous findings rather than providing a more complete understanding of the population's differing species.Comparative genomics therefore provides a significant leap forward in understanding the relationships within the species complexes; the designation of the species within these complexes is less straightforward, and will likely need to be determined on a case by case basis, by a consensus of scientists and clinicians, rather than by phylogenetic algorithms.

Clinical implications of nomenclature

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Cryptic species, identified as monophyletic clades, have been discovered by DNA sequencing in several medically important fungal pathogens, including *Cryptococcus neoformans* and *Cryptococcus gattii*. Discovery of a cryptic species is often accompanied by differences in geographic distribution, as one might expect for organisms with a reservoir in nature. Divergence arises biologically over centuries to meet the demands of the local ecology. Clades may or may not differ in the infections which they cause and this distinction has implications for medical practice.The rapid, recent changes in medicine have led to an increased reliance of clinicians on the medical literature for guidance. Nomenclatural instability disrupts vital connections in the medical literature by providing information that may change between studies, lessening reproducibility of clinical data. Indeed, reproducibility in scientific findings has been an area of active concern recently, as small sample or single study-based conclusions have proven poorly reproducible, with important implications regarding clinical practice. Unless the clinician's understanding of pathogenesis, diagnosis and management is significantly and reproducibly altered by, in this case, giving a cryptic species a new name, ambiguities in the significance and ramifications of such changes could result in problematic clinical care. An alternative to changing the species name of a clade could include giving a clade a number or MLST type, pending the acquisition of clinically significant differences concluded from multiple independent studies. As information about the clade accumulates, clinical impact may be found that justifies distinguishing this clade as a new species.

Historical review of cryptococcal nomenclatural changes

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The names of the disease cryptococcosis and its etiologic agents had remained chaotic for the first half century since discovery of both the disease and its etiologic agents in 1894. The disease had six different names and the etiologic agent had 37 synonyms classified under seven different genera. Benham clarified the confusion by comparative studies on pathogenic vs non-pathogenic species of the genus *Cryptococcus* and called the disease cryptococcosis and described its etiologic agent as the single species, *C. neoformans* in 1950. Twenty five years later, discovery of the two teleomorphs among the strains of *C. neoformans* eventually led to the recognition of two distinct etiologic agents: *C. neoformans* and *C. gattii*. Subsequently, whole genome sequence comparisons between representative strains of *C. neoformans* and *C. gattii* has confirmed that the genetic hiatus between the two species is wide enough to be different species with only 85.6-87% in chromosome sequence identity.The two species system had been widely accepted since 2002 but with an understanding that each species contains several genetically diverse clades.

Hidden in plain sight: Mechanism of *C. neoformans* immune evasion

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Like many fungal pathogens, *Cryptococcus neoformans* is particularly adept at avoiding recognition by the immune system. The polysaccharide capsule shields the cell surface from host immune surveillance. Additionally, the cell wall plays an additive role in this shielding process. In response to host signals, the fungal cell wall is actively organized to hide more immunogenic molecules as well as to expose those components required to efficiently bind capsule.Lastly, this fungus' ability to effectively enter into and replicate within phagocytic host immune cells also contributes to its survival with minimal resulting immune activation.We have identified that the fungal-specific Rim signaling pathway mediates the remodeling of the cryptococcal cell wall in response to host signals. Activated by alkaline pH and other cell stresses, this pathway directly controls the expression of multiple genes required for cell wall biosynthesis and modification. Mutations in Rim signaling result in disorganized cell walls, exposure of highly immunogenic epitopes, and enhanced inflammation in animal models. Although some components of the Rim signal transduction pathway are highly conserved among diverse fungal species, we have determined that the upstream Rim sensing/activation complex utilizes novel means to sense and response to host pH signals. These novel components include lipid elements in the cell membrane and downstream scaffold/chaperone proteins. Together, these studies inform how microorganisms might respond to fundamental environmental signals such as changes in extracellular alkalinity. Additionally, we are now poised to better dissect the roles of specific cell wall components on microbial immune avoidance and recognition.

Pathogenesis and Germination of Spores

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Given their small size, resistance properties, and disease-causing ability in animal models, spores of *Cryptococcus* are a likely cause of natural infections in humans. Spores are the products of sexual development, which appears to occur in the environment and can be carried out in the laboratory. Using pure populations of spores, we determined that spores interact fundamentally differently than yeast with the mammalian immune response, lead to higher fungal burdens in the brain, and are more likely to cause signs of central nervous system disease in mice. The properties of spores that facilitate these differences are largely unknown; however, it is evident that spores must germinate into vegetatively growing yeast to colonize new environments, including mammalian hosts. As such, understanding how germination occurs is key in understanding spore-mediated infection and disease. In previous proteomic and genetic studies we determined that proteins enriched in spores are integral to sexual development and not simply related to general cellular processes (e.g. cell division or stress responses). Thus, understanding the links between development and germination can shed much-needed light on spore survival and generate tools to understand the properties and pathways of spores that make them effective infectious particles. The process of germination in *Cryptococcus* leads to measurable changes in spore size and shape that correlate with changes in surface carbohydrates and protein composition. Capitalizing on these properties, we developed two quantitative assay systems to monitor germination. The first, a luciferase-based expression system, tracks the expression of yeast- and spore-enriched proteins as markers of germination stages. This assay has been used in high throughput screening of small molecules libraries to identify germination inhibitors. From a 75,000 compound library, we identified 111 inhibitors of spore germination that fall into discrete structural categories. These compounds will be assessed in the second assay system in which we use pipette-accessible microfluidic devices and automated microscopy to track and quantitate populations of spores undergoing germination. This assay capitalizes on the synchronous germination profile of *Cryptococcus* spores and the differences in size and shape between spores and yeast. It allows the quantitative assessment of germination of hundreds of spores over time at both the single-cell and population levels. Using this assay, we tested the effects of known antifungal agents, uncharacterized compounds, and genetic mutants to probe their effects on both morphological and molecular events of germination. With this approach, we have identified discrete morphological and cellular events that occur during germination that correlate with changes in specific molecular pathways. Based on these findings, we hypothesize that the process of germination is molecularly distinct from vegetative growth and is controlled by discrete checkpoints. Our data provide the first glimpses spore-specific components and pathways that inform fundamental pathogenic spore biology and provide potential targets for inhibition. Inhibition of spore germination has implications for *Cryptococcus* survival in host environments and provides opportunities for the development of prophylactic antigerminant therapeutics for use in vulnerable patient populations.

Roles for proteostasis, nutritional adaptation and trafficking in Cryptococcal virulence

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Adaptation of *C. neoformans* to the host environment depends on recognition and exploitation of nutritional signals. We are using a variety of approaches (proteomics, transcriptomics, mutant screens, and targeted gene deletion) to identify and characterize functions involved in adaptation.For example, our recent proteomics studies identified the proteosome as a downstream target of regulation by cAMP-dependent protein kinase (PKA). Subsequent use of proteosome inhibitors and mutant screens confirmed a connection between the proteosome and capsule formation. These approaches also identified genes encoding functions for proteostasis that interact with PKA signaling and that impact capsule formation. Because capsule elaboration and virulence are also influenced by iron availability, we are pursuing parallel studies to identify iron sensing and uptake functions. An emerging theme is that functions for intracellular trafficking often play roles in both capsule export and iron import. We have demonstrated this theme through characterization of the role of the ESCRT pathway in both heme acquisition and capsule elaboration. More recently, we extended this work through the identification of mutants with altered susceptibility to non-iron metalloprotophoryins. These inhibitors may hold promise as novel antifungal drugs that impede iron acquisition from heme during infection.

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Population genomics and the evolution of virulence traits in *Cryptococcus neoformans*

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To characterize the population diversity, subdivisions, and the level of genetic exchange, we sequenced the genomes of ~400 diverse isolates of *Cryptococcus neoformans* var *grubii* (serotype A). While our data supports the three previously identified lineages (VNI, VNII, and VNB), phylogenetic analysis highlighted a deep, non-recombining split in VNB (VNBI and VNBII) and that VNI was further subdivided into three distinct clades, two of which were globally distributed and one of which was restricted to sub-Saharan Africa. Tracing the history of *C. neoformans* var *grubii* using population genomic and Bayesian coalescent analyses estimated the timing of VNBI and VNBII diversification during expansion of the African savannah, followed by population bottlenecks and subsequent diversification and global dispersal of VNI. Despite the higher prevalence of *MAT***a** isolates in VNB compared to VNI, we find similar levels of linkage disequilibrium in VNI, VNBI and VNBII. While we did not detect recombination between lineages based on genome wide comparisons, we identified introgressions (5 to 260 kb)in all pairwise combinations of VNI, VNBI, and VNBII as recipient and donor.We find unique evolutionary trajectories for mating type loci, which suggest local introgression of the *MAT***a**locus from VNBI into VNI. Notably, some haploid isolates show more widespread hybrid ancestry of multiple lineages, including isolates that appear to have originated from recent interbreeding.Many isolates display evidence of aneuploidy; in diploid isolates both of serotype A/A and A/D such aneuploidies have resulted in loss of heterozygosity, where a chromosomal region is represented by the genotype of only one parental strain.By assembling and annotating multiple isolates of VNI, VNII, and VNB, we find that gene content is highly conserved, with few examples of lineage-specific genes. Rapidly evolving genes between the lineages include transcription factors and transferases, many of which have been implicated in virulence or oxidative stress resistance. To evaluate how selective pressures in the environment coincidentally adapted *C. neoformans* for human virulence, we focused on a set of clinical and environmental VNB isolates from sub-saharan Africa. We found that the VNBIIsublineage was enriched for clinical samples relative to VNBI, while phenotypic profiling of sequenced isolates demonstrated that VNBI isolates were significantly more resistant to oxidative stress and more heavily melanized than VNBII isolates. Lack of melanization in both lineages was associated with loss-of-function mutations in the *BZP4* transcription factor. A genome-wide association study across all VNB isolates revealed sequence differences between clinical and environmental isolates in virulence factors and stress response genes. Inositol transporters and catabolism genes, which process sugars present in plants and the human nervous system, were identified as targets of selection in all three lineages. These data highlight the complex evolutionary interplay between adaptation to natural environments and opportunistic infections, and that selection on specific pathways may predispose isolates to human virulence.

Azole heteroresistence in *Cryptococcus*

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Fluconazole (FLC) has been the major drug for treatment and long term maintenance therapy of cryptococcosis,especially in AIDS patients. However, *Cryptococcus* strains are innately heteroresistant to fluconazole in vitro and in animal models. Heteroresistanceis the emergence of a resistant minor subpopulation that can tolerate higher FLC concentrations than the strain's MIC. It has been shown that heteroresistant clones contain disomic chromosomes. The mechanism of how aneuploidsareformed upon FLC treatment has remained unclear. When cryptococcal cells are exposed to high concentrations of FLC, few cells containing multiple nuclei appear within a few hours. It is feasible that the extra copy of a disomic chromosome in the resistant clones may have resulted from non-disjunction after fusion of multiple nuclei. In contrast to this hypothesis, however, multinucleated cells did not yield higher frequencies of heteroresistant clones than uninucleate cells. To further monitor the nuclear behavior of the cells upon FLC treatment, we labeled the nuclei by tagging histone H2Bwith GFP. Cells were observed at 10 minutes interval under microscope in the presence of FLC for three days. Although the frequency of FLC resistant colonies occurred no more than 1%, we were able to identify and follow a few cells that proliferated for three days and form colonies by tolerating FLC stress.All these colonies were found to have been derived from uninucleatemother cells. Neither of the surviving colonies derived from cells containing multiple nucleinor from a nuclear fusion event.Furthermore, the results of fluctuation test did not support spontaneous mutation for FLC resistant clones. Although we could not rule out the possible existence of transient and extremely unstable aneuploid cells that could not be distinguished under microscope among the FLC untreated parent cells, we propose that the aneuploid formation in FLC resistant clonesisthe result of de novo chromosomal duplication.

Epidemiological cutoff values (ECVs) in *Cryptococcus neoformans /Cryptococcus gattii* species complex

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There are no breakpoints for any antifungals against any *Cryptococcus* species, and because of the lack of controlled trial clinical outcome data, there probably never will be. Epidemiological cutoff values, while not a substitute for breakpoints, serve to define wild type and non-wild type isolates of a given species against a given antifungal agent. The epidemiological cutoff value (ECV) defines the end of the wild type natural distribution of MIC values and in so doing allows for the identification of isolates that may contain mutations that confer antifungal resistance. The Clinical and Laboratory Standards Institute recently defined epidemiological cutoff values for a number of species in the *Cryptococcus gattii* / *Cryptococcus neoformans* species complex against the azoles as well as flucytosine and amphotericin B. The ECVs were species-specific, with those in the *C. gattii* species complex being higher than those on the *C. neoformans* species complex and *C. deuterogattii* (*C. gattii* VGII) being higher than the other *C. gattii* species complex isolates. In this talk the limitations and the potential clinical usefulness of ECVs for *Cryptococcus* will be discussed.

The R&D of new antifungals targeting the synthesis of fungal sphingolipids

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In recent years, it has been reported that fungal glucosylceramide (GlcCer) is required for virulence of many fungi, including *Cryptococcus neoformans* (*Cn*),*Candida albicans* (*Ca*),*Aspergillus fumigatus* (*Af*)and others. In addition, GlcCer is detected only in the host infective form (yeast) and not in the environmental form (mold) of many dimorphic fungi. Furthermore, the synthesis of GlcCer seems to be important also for *Pneumocystis* pneumonia (PCP) as glucosylceramide synthase transcripts are highly elevated at the time of isolation of the fungus from a fulminate lung infection. Studies in our and other labs revealed that GlcCer promotes alkaline tolerance in fungi. Particularly, GlcCer regulates fungal cell replication by promoting cell cycle progression and cytokinesis in a neutral/alkaline but not acidic environment. Taken together, these studies suggest that GlcCer is most likely a pan-fungal virulence factor required during infection to promote fungal growth at neutral/alkaline environments (e.g. alveolar spaces and bloodstream), and as such, it is a promising novel drug target. Therefore, we looked for inhibitors of GlcCer synthesis by screening a ChemBridge library for compounds that inhibit fungal growth in an environment similar to the lung and bloodstream: neutral/alkaline pH, 37ºC and 5% CO_2 using *Cn* as a model organism. We identified 2 compounds (BHBM and its derivative D0) that significantly decreased the synthesis of GlcCer in *Cn* but not in mammalian cells. The compounds are fungicidal and able to improve mice survival during invasive cryptococcosis, candidiasis and pneumocystosis. Mechanistic studies show that the compounds target the fungal vesicle trafficking, which is how ceramide is transported for the synthesis of GlcCer. Screening of additional derivatives identified very potent antifungal compounds with a selectivity index >4,000. This presentation highlights the discovery and the future research and development of this new class of antifungals.

New Antifungal Drugs and Implications for Treatment of Cryptococcosis

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The treatment of life-threatening cryptococcal infections, particularly those involving the central nervous system, has traditionally relied heavily on the use of parenteral formulations of amphotericin B, with or without adjunctive therapy with 5-flucytosine or fluconazole. The management of severe cryptococcal infections not involving the CNS usually reflects an aggressive approach similar to that used for CNS infections. The treatment of less severe forms of cryptococcosis, including focal pneumonia and disease limited to the skin, typically involves fluconazole alone in the appropriate clinical setting. Virtually all of the prospective controlled studies evaluating treatment of cryptococcosis have included exclusively patients with CNS cryptococcosis, with or without extra neural involvement. There are no prospective randomized studies evaluating the treatment of non-CNS cryptococcosis. The original studies evaluating treatment for cryptococcal meningitis involved almost exclusively non-HIV infected patients. However, in the last 25 years the vast majority of published prospective studies have examined various treatment approaches among HIV infected patients with cryptococcal meningitis. It is largely based on the findings of these robust studies that we have derived our current therapeutic approach to cryptococcal meningitis. Indeed, recent data confirm that the most effective and rapidly fungicidal regimen for cryptococcal meningitis as well as use of amphotericin B plus flucytosine as induction therapy for at least the first 14 days following diagnosis followed by oral fluconazole. Among the newer expanded-spectrum azoles, including voriconazole, posaconazole and isavuconazole, there are limited in vitro data and few clinical observations which provide much insight into their potential roles for treatment of cryptococcosis. In most circumstances, these are considered second-line agents in situations where toxicity or fluconazole resistance has become a concern. In the ongoing *C gattii* outbreak in the Pacific Northwest, many of these isolates are relatively non-susceptible to fluconazole, yet retain low MICs to posaconazole, thus posaconazole is used frequently in this setting despite the lack of compelling clinical data to support this alternative approach to therapy. Among the newer antifungal agents that are being developed, many have superb in vitro activity and in animal models of CNS cryptococcosis. Selected orally available CPY 51 inhibitors (VT 1129, VT 1598) provide outstanding in vitro activity and in animal models of CNS cryptococcosis, comparing very favorably to AmB with respect to rapid sterilization of brain tissue. Similarly, a cochleate formulation of AmB (Matinas) has demonstrated excellent acitivity in an animal model of CNS cryptococcosis. Other novel antifungal agents also have potential in this arena. The use of existing adjunctive agents, such as calcineurin inhibitors and IFN gamma, may play increasing important roles as adjunctive agents for treatment of CNS cryptococcosis as we learn more about the biology of the organism and specific defects in host defense. Finally, the physical removal of viable cryptococci and cryptococcal antigen through a novel device may offer significant survival benefit in the setting of acute CNS cryptococcosis.

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The blood-brain barrier and beyond: insights from multicellular and animal models

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The passage of cryptococci across the blood-brain barrier (BBB), establishment of infection via penetration into the perivascular space and subsequent entry into the subarachnoid space to cause meningitis have been the subject of considerable research. It has been established in both *in vitro* and *in vivo* models that cryptococci can cross the BBB directly by transmigration through the endothelial cell layer of the post-capillary venules within the cerebral microvasculature. Furthermore, there is persuasive evidence for transmigration within mononuclear phagocytes, via the so-called Trojan Horse mechanism though whether this also occurs via diapedesis through intact endothelial cells has not been established.Furthermore, there is evidence from mouseand rat models and in human diseasethat the two clinical type strains of *C. neoformans* (H99) and *C. gattii* (R265) are differentially neurotropic though these observations require rigorous experimental validation and mechanistic studies, both *in vitro* and *in vivo.*Our work has focussed on the BBB as the portal of entry of cryptococci into the CNS and a potential contributor to differential neurotropism of H99 and R265.Our initial studies were performed with a well-validated *in vitro* model of the BBB,that utilizes a monolayer of the hCMEC/D3 cell line derived from human brain endothelial cells (HBEC), in a standard Transwell system.To better recapitulate the anatomical organisation of the BBB *in vitro* we have developed a tri-cell model, to be described in this presentation. We have also developed a highly reproducible C57Bl/6 mouse model that overcomes the need to use likely non-pathophysiological high concentrations of cryptococci to demonstrate transmigration *in vivo,* by inoculation of as few as 5,000 cryptococcal cells into the retro-orbital plexus. *Results:* Using macrophage-like THP-1 cells in the HBEC monolayer BBB model, we showed that: (1) Phagocytosis of H99 by THP-1 cells was greater than that of R265; (2)H99 and R265-loaded THP-1 bound similarly to TNF-activated HBECs under shear stress; (3) more H99-loaded macrophages were transported across an intact HBEC monolayer; (4)H99was expelled from transmigrated THP-1 at a higher rate than R265[1]. Taken together, these findings suggest that increased transmigration of H99 across the BBB by the Trojan Horse mechanism, with increased extrusion from mononuclear phagocytes post-migration could contribute to the apparently higher neurotropism of *C. neoformans.* Recapitulation of these studies in a tri-cell model comprised ofHBEC, astrocytes and pericytes is underway. Longitudinal dose ranging studies (survival and CNS cryptococcal burden) were performed*in vivo*. C57Bl/6 mice inoculated with 5,000 cfu of H99 survived for an average of 10 days and developed para-vascular cryptococcomas with a surrounding cellular response. In contrast, R265-inoculated mice survived for a median of 26 days (range 18-53), developed fewer, smaller cryptococcomas and the brain fungal load at day ten was two logs lower. Notably, over time, H99 burdens in blood increased whereas with R265 infection, blood cultures remained negative.Prominent accumulation of leukocytes (both neutrophils and mononuclear phagocytes) causing vascular compression was evident in perivascular spaces within the brain at day 10 in H99-infected mice and wasassociated with a transient increase in pro-inflammatory cytokines. In mice infected with R265, the perivascular infiltrate was much less, commensurate with no increase in pro-inflammatory cytokines. In chimeric (Macgreen) mice infected with H99 we used fluorescence microscopy and differential monocyte/microglial markers to confirm the haematogenous origin of the perivascular mononuclear phagocytes, 4% of which contained intracellular cryptococci and a smaller number of neutrophils, 8% of which contained intracellular cryptococci (typically one per phagocyte). We conclude that differences in neurotropism of H99 and R265 strains of *C. neoformans* and*C. gattii* are manifest (at least in part) at the level of the BBB.
Reference : Sorrell TC, Djordjevic JT, Francis K, Dietmann A, Milonig A, Casteneda D-A, Selevtsova E, Combes V, Grau, GER. Cryptococcal transmigration across a model brain blood-barrier: evidence of the Trojan horse mechanism and differences between *Cryptococcus neoformans* var. *grubii* strain H99 and *C. gattii* strain R265. *Microbes & Infection* http://dx.doi.org/10.1016/i.micinf.2015.08.017 2015

How *Cryptococcus neoformans* interacts with blood-brain barrier

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Globally, nearly a million cases of cryptococcal meningoencephalitis occur each year, resulting in 624,700 deaths by 3 months after the occurrence of diseases. Sub-Saharan Africa had the highest yearly burden estimate, while Western and Central Europe and Oceania had the lowest. Without antifungal management, mortality of cryptococcal meningoencephalitis is 100% within the first two weeks of hospitalization. To cause meningoencephalitis, *Cryptococcus* must transverse the blood-brain barrier (BBB). The BBB prevents most molecules from passing into the brain, but it allows transporters to provide energy for brain metabolism. If *Cryptococcus* hijacks any of these transporters or their signals, it will be able to cross the BBB and cause central nervous system (CNS) infection. Much of this understanding has been achieved with murine models and human brain microvascular endothelial cells (HBMECs).The first step for establishing cryptococcal meningoencephalitis is the association of *Cryptococcus* cells with HBMECs. The dynamic interactions may represent events during the adhesion and entry of *Cryptococcus* at HBMEC membrane lipid rafts by stimulating downstream events of the entry process. Subsequently, membrane signaling may relay through CD44 to the act in cytoskeleton inside the HBMECs. Ultrastructural visualization of scanning electron microscopic images disclose the progression of invasion: internalization by the microvilli embrace of *Cryptococcus*, followed by a zipper-like mechanism in which the hosts' cell plasma membrane encompasses the invading yeasts. This transcellular mechanism needs *Cryptococcus* cell-induced HBMEC cytoskeletal rearrangements for the gathering of actin at the site of *Cryptococcus* entry.The *Cryptococcus* cell may then be drawn gradually into the host cell.*Cryptococcus*, however,is not limited to one method of entry. Paracellular pathway and Trojan horse route by professional phagocyte are critical in cryptococcal brain invasion.Several cryptococcal genes related to the traverse BBB are identified, such as *CPS1*, *URE1*, *FNX1*, *RUB1*, *PLB1*, *MPR1*, *ITR1a* and *ITR3c*. *Cryptococcus neoformans* produces extracellular microvesicles (CnMVs) that cross the fungal cell wall to enter the extracellular space not only to provide polysaccharides for capsule assembly but also to deliver various factors to host tissues.CnMVs could fuse with HBMECs in two steps, including pre-adhesion and afterwards adhesions. The secreted 14-3-3-GFP molecules from CnMVs in infected brain are suggested as a marker of microvesicles.Although the CnMVs contain several known virulence factors, exactly which components are involved in adhesion to HBMECs is currently unknown.Given the plethora of roles associated with microvesicles in different organisms, it is conceivable that CnMVs have many yet to be identified pathogenic roles. Investigation of the genetic and molecular mechanisms for cryptococcal neurotropism may lead to better understandings of how *Cryptococcus* exclusively crosses the BBB. With this knowledge, we may be able to improve strategies to disturb this yeast's ability to enter CNS.

Brain infection by *Cryptococcus neoformans*: What we learned from intravital imaging

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Cryptococcus neoformans is an encapsulated budding yeast that causes a life-threatening illness in immunocompromised individuals. Although the infection starts in the lung, cryptococcosis commonly presents as meningoencephalitis, which is one of the most common infections of the central nervous system and a leading cause of death in HIV-infected individuals. Hematogenous dissemination of *C. neoformans* is one of the most critical steps in the development of meningoencephalitis. We became interested in a number of questions related to the pathogenesis of cryptococcal meningoencephalitis. (1) How is *C. neoformans* circulating in the loodstream arrested in the brain vasculature prior to transmigration? (2) How does the arrested *C. neoformans* invade the brain across the blood-brain barrier (BBB)? and (3)How do circulating leukocytes respond to *C. neoformans* arrested in the brain vasculature? As the interaction of *C. neoformans* with brain endothelial cells or circulating immune cells is a transient and dynamic process, we developed an in vivo model system based on intravital microscopy (IVM) to address these questions. We demonstrated that *C. neoformans* moved at the same speed as the blood and came to a sudden stop in the capillary bed of the brain, in the same manner and with the same kinetics as polystyrene microspheres, without rolling and tethering to the endothelial surface. The yeast cells were mainly arrested in capillaries, which was not affected by viability or known virulence factors. These results suggest that *C. neoformans* is mechanically trapped in the brain, which raises novel challenges for therapies to avoid arrest. Following arrest in the brain, *C. neoformans* was directly seen to cross the capillary wall of living animals in real time. In contrast to trapping, viability was required for *C. neoformans* to cross the BBB. Urease is critically involved in brain transmigration of the organism. Accordingly, a urease inhibitor could ameliorate infection of the mouse brain by reducing transmigration of *C. neoformans* into the brain. As neutrophils are the most abundant circulating phagocytes, we next examined the dynamics of interactions of neutrophils with *C. neoformans*. Using in vitro live-cell imaging, neutrophils were directly seen to move toward *C. neoformans* and then rapidly internalize the yeast. Complement C5a–C5aR signaling was essential for phagocytosis of *C. neoformans* by neutrophils by guiding their migration to the yeast cells and enhancing surface expression of CD11b. Furthermore, the p38 MAPK pathway, but not the Erk pathway, was critically involved in C5a–C5aR-mediated chemotaxis of neutrophils. We also observed in vitro neutrophil swarming toward *C. neoformans*, which is mediated by complement and Leukotriene B4. With the use of IVM, we further demonstrated that neutrophils crawled to the yeast cells that had been arrested in the brain microvasculature. Following interactions *with C. neoformans*, neutrophils were seen to internalize the organism and then circulate back into the bloodstream, resulting in a direct removal of the organism from the endothelial surface before its transmigration into the brain parenchyma. Depletion of neutrophils enhanced brain fungal burden, while enhancing the recruitment of neutrophils improved intravascular clearance of *C. neoformans* in the brain. Taken together, our studies suggestthat *C. neoformans* is mechanically trapped in the brain capillary, but actively transmigrates to the brain parenchyma with contributions of urease. Neutrophils have the ability to remove *C. neoformans* directly from the brain vasculature in a "vacuum-cleaner" type of behavior.

Progress towards cryptococcal vaccine development

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The work described here is focused upon the preclinical development of vaccines to protect at risk populations from cryptococcosis. For these studies, we have been using glucan particles (GPs) as a combined delivery system and adjuvant. GPs are purified, porous cell wall shells derived from baker's yeast and composed primarily of β-1,3-glucan. In vivo, phagocytosis of GPs is mediated by complement receptors and Dectin-1. Polymer-complexed cores can be constructed within the hollow GPs, enabling the delivery to phagocytes of a wide variety of "payload" classes including proteins. Subcutaneous immunization of mice with GPs containing complexed ovalbumin results in robust and long-lasting antigen-specific humoral and CD4 T cell (Th1- and Th17-biased) responses. To extend these studies to vaccines against cryptococcosis, vaccine candidates were prepared consisting of GPs containing complexed alkaline extracts derived from Cn or Cg. Vaccination of mice with these preparations resulted in partial protection against lethal challenge with Cn or Cg. To identify the immunoprotective antigens responsible for protection, we have been taking biased and unbiased approaches. The biased approach has been to recombinantly express antigens (rAgs) known to be immunoreactive. For the unbiased approach, the proteome of the protective extract was defined by electrospray ionization liquid chromatography tandem mass spectrometry (LC-MS/MS). Then, the top antigens based on abundance are being recombinantly expressed. rAgs identified by both approaches have been loaded into GPs, following which mice were vaccinated and then challenged with Cn or Cg. Thus far, nearly 20 rAgs have been tested in this fashion; three have been identified as candidate antigens for further study. Protection elicited by rAgs varies as a function of the mouse strain and differs when comparing Cn and Cg. Mice that lack T cells are not protected but B cell deficient mice are. Some rAg combinations exhibit enhanced protection. Future studies will examine ex vivo antigen-specific responses in humans with cryptococcosis

Evasion of Immune Response by Highly Virulent *Cryptococcus gattii*

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Epidemiology of cryptococcosis in Japan. Disseminated cryptococcosis is a serious infection which occurs in healthy individual, and potent domestic infection by *Cryptococcus gattii* had been reported also in Japan. Considering seriousness and public impact of this infection, disseminated cryptococcosis has been listed as a notifiable disease since Oct. 2014 in Japan. National surveillance revealed that confirmed disseminated cryptococcosis was documented at any places of the nation with average occurrence rate of 0.1 per 100,000/year. We studied in pathogenicity of domestic isolates of *C. gattii* and a potent prophylactic strategy for the disease.Mice survival and histopathological study. We focused on JP02 strain that showed the highest killing potency in a mice cryptococcosis model as well as R265 strain. JP02 was classified in VGIIc by MLST. Histopathological examinations of the lung of the same model revealed that inoculation with JP02 induced a weaker protective inflammatory response in the murine lung than H99. This finding suggested impaired pathogen recognition in JP02-infected mice. Dendritic cells (DCs) response to JP02. We tested whether mouse JAWSII DCs could recognize live H99 and JP02 cells in co-culture by measuring IL-6 released into the culture supernatant. Whereas live H99 cells stimulated IL-6 production from JAWSII DCs, live JP02 cells did not. However, it is possible that live JP02 cells suppress IL-6 production by cytotoxicity or by suppressive signals. To examine the first possibility, we cultured JAWSII DCs with heat-killed JP02 cells or H99 cells. Although heat-killed H99 cells still induced IL-6 production from JAWSII cells, heat-killed JP02 cells did not. Further, IL-6 production induced by H99 cells was not suppressed by co-inoculation with JP02 cells. These results suggest that JP02 is not recognized by JAWSII DCs and that the virulent *C. gattii* possesses molecular mechanism to evade recognition by host innate immune cells. IL-6 induction of capusular and extracellular polysaccharide. We extracted capsular polymeric substances (CPS) and extracellular polysaccharide(EPS) from H99 and JP02 cells, and applied them to JAWSII DCs. The CPS extract from H99 cells induced IL-6 production from JAWSII DCs, on the other hand, CPS extract from JP02 cells did not induce measureable IL-6 production. Results with EPS was similar to those with CPS. These results suggest that JP02 EPS are not recognized and so do not induce cytokine release from DCs. Difference in extracellular polysaccharide structure. Monosaccharide analysis was performed by high performance liquid chromatography (HPLC). The EPS fractions from H99 and JP02 cells showed similar elution profiles and contained one major acidic subfraction which represented glucuronoxylomannan (GXM). There were only small differences in the monosaccharide composition between H99 GXM and JP02 GXM; specifically, the xylose content of JP02 GXM was higher than that of H99 GXM. Proton nuclear magnetic resonance (1H NMR) analysis showed that the major difference between the spectrum of H99 GXM and JP02 GXM was the pattern of methyl protons indicating O-acetyl groups. Since IL-6 production was eliminated by deacetylation, O-acetylation pattern of GXM in *C. gattii* may be involved in induction of inflammatory cytokine production from dendritic cells

The front line of anti-cryptococcal defense: The complex roles of macrophages and dendritic cells

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Upon inhalation into the lung,*Cryptococcus neoformans* initially interacts with innate cells in the pulmomary tissues, including macrophages and dendritic cells (DCs). We have shown that DCs kill *C. neoformans*, and we have identified lysosomal enzymes that involved in cryptococcal killing. In contrast, several studies have shown that macrophages can either kill *C. neoformans* or allow intracellular replication of the organism. Several laboratories have examined the cryptococcal-associated factors that promote intracellular cryptococcal growth in macrophages, but few studies have characterized macrophage factors that govern cryptococcal killing versus cryptococcal growth. Recent publications have suggested that in vitro primary macrophage cultures can result in mixed populations of macrophages. Therefore, we examined the anti-cryptococcal activity of two subsets of human PBMC-derived macrophages. We differentiated primary human macrophages from human PBMCs using M-CSF and separated two populations of macrophages using human macrophage cell surface markersCD11b and CD163. We found that $CD11b^+$ human macrophages inhibited cryptococcal growth, while $CD163^+$ human macrophages allowed cryptococcal replication and growth. Imaging flow cytometry studies showed that $CD11b^+$ macrophages contained crescent-shaped, killed *C. neoformans* cells while the $CD163^+$ macrophages contained budding, replicating cryptococcal cells. These different subsets of human macrophages can now be used as a model system to examine the mechanisms in macrophages responsible for killing compared to intracellular cryptococcal growth. These studies suggest that intracellular cryptococcal growth is dependent not only on cryptococcal virulence factors, but also on the activity of phagocytes that interact with the organism.

Natural Killer cells recognize, are activated by, and kill *Cryptococcus*

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The host, desperate to survive cryptococcal invasion, employs important but extremely complex mechanisms of host defense. One such mechanism involves NK cellreceptor-mediated recognition of fungi. Multiple receptor ligation is followed bya cascade of protein phosphorylation eventsthat are part of intracellular signal transduction. This results inNK and fungal cell conjugate formation with development of an NK and fungal cell synapse. Spatial and temporal positioning of the cytolytic payload in secretory lysosomesare transported via molecular motors on microtubules to align with the microtubule organizing center and come into apposition with the NK cell plasma membrane in the region of the fungal cell synapse. There, the cytolytic cargo is deployed and the fungal cell membrane receives a fatal blow. Recent advances in live cell imaging and the distinct elements of NK cell-mediated fungal killing permit novel insights into the mechanisms by which NK cells exert their cytotoxic potential as well as important insights into fungal host defense.

Regulation of titan cell formation in *Cryptococcus neoformans*

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Cryptococcus neoformans is a unique yeast due to its ability to adapt to the lung environment and persist in this organ. There are several factors that contribute to fungal survival. The capsular polysaccharides are virulence factors that impair the host response. Furthermore, the capsule confers protection against some of the challenges of the immune cells, such as reactive oxygen species. But one of the most characteristic features of this fungus is its ability to develop morphological changes that confer a selective advantage to the pathogen. The main change is produced by a significant increase of the size of the fungal cells, which can be achieved by a massive enlargement of the capsule, or by an increase of both the cell body and capsule. This last case results in the appearance of cells of an abnormal large size, which have been denominated as titan cells. Our group is interested in the characterization of this morphological transition, and in particular, in the study of the factors that induce titan cell formation in *C. neoformans*. We have recently identified new factors that regulate this transition in vitro. In particular, addition of mammalian serum to media with a low concentration of nutrients results in a significant increase of cell size that partially reproduced the phenomenon found in vivo. This process is enhanced by CO_2 andoxygen limitation, which simulate the conditions found in the lung. We also observed that formation of titan cells is regulate by cell density and is repressed by some quorum sensing molecules, such as farnesol. RNAseq experiments have revealed that titan cells significantly overexpressed membrane proteins and transporters. Our results have allowed us to suggest an integrated model of different factors and pathways that are induced during titan cell development. Another interesting aspect is that the morphology of cryptococcal cells varies depending on the host genetic background, and that Th2-type responses correlate with a higher proportion of titan cells in the lungs. We are investigating how the immune response regulates the growth of *C. neoformans* cells size. We have determined that CD4 T cells play an important role in controlling cryptococcal cells size through a mechanism that is still under investigation. These data will contribute to understand how cryptococcal cells differentially response and adapt to different types of hosts.

Diversity in yeasts and host responses during murine cryptococcosis

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During infection and upon macrophage interaction, Cnis known to adapt through various mechanisms generating in situ fungal diversity: morphological modifications with formation of Titan cells, intracellular multiplication,capsule modifications, or phenotypic switching, and up-regulation of genes involved in resistance to stress, or nutrient deprivation.During murine cryptococcosis and macrophage uptake, stress and host immunity induce *C. neoformans* heterogeneity with generation of a sub-population of yeasts that has been shown to harbour a phenotype compatible with dormancy (low stress response, latency of growth. Dormancy in *C. neoformans* had been suspected by clinical and epidemiological data and evidenced by genotyping results a few years ago. It is known that metabolically quiescent pathogens can persist in a viable non-replicating state for months or even years, leading to latent infections and sometimes to reactivation.Dormant sub-population of yeasts has been shown to harbour up-regulated mitochondrial activity, phenotype considered as a hallmark of quiescence in stem cells. Based on these findings, a new in vitro model was implemented and standardized in our lab. The diversity translates also into variable outcomes and host response after intravenous inoculation with different strains. Survival was associated with more antibody production against an aspartyl-protease Pep1. We thus studied in vitro(phagocytosis, growth curves) and in vivo (survival, fungal burden, brain lesions, inflammatory response) the role of PEP1 (active immunization or serotherapy with anti-Pep1 monoclonal antibodies) in the course of murine cryptococcosis and whether this role differs with the infecting strain (H99/52D).

Dissecting the pathway regulating cryptococcal vomocytosis

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The two pathogenic species of Cryptococci, *Cryptococcus neoformans* and *C. gattii*, share a remarkable ability to evade the innate immune system and disseminate throughout the body. This is thought, in large part, to be the result of natural selection through environmental amoebae, since virulence traits that the fungus has evolved to survive within such predators typically work just as effectively within human phagocytes. In this talk I will discuss our recent work in probing the cryptococcal/macrophage interaction. In particular, I will discuss what we have learned about the molecular basis of "vomocytosis", a phenomenon that the pathogen uses to exit from phagocytic cells. In addition, we are also interested in the genetic changes that drive hypervirulent outbreaks of cryptococcosis in otherwise healthy individuals and I will attempt to "compare and contrast" these disease situations and speculate on what they tell us about the innate immune response to fungal pathogens more generally.

Progress in Early diagnosis and identification of Cryptococcosis: "The Asia-Pacific Perspective"

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A diagnosis of cryptococcosis could be readily made with basic laboratory methods. Generally, a gold standard for diagnosis of infection is an isolation of a live organism from the site of lesion. Therefore, an isolation of *Cryptococcus neoformans*/*gattii* from an infection site is important for a diagnosis of cryptococcosis. However, the cryptococcal isolation, including identification, takes at least 48-72 hours. A rapid test, capsular antigen detection, shows up to 100% specificity and sensitivity, especially when tested with cerebrospinal fluid from the most common site of infection, meninges. This test is readily available as commercial kits based on latex agglutination and lateral flow methods. The superb performance of the antigen detection methods makes nucleic acid detection unpopular in most cases of cryptococcosis. Lastly, a bedside microscopic direct examination, an India ink preparation, shows high sensitivity, approximately 80%, in AIDS patient with cryptococcosis. On the other hand, an identification of *Cryptococcus spp*. to a species level is lacking in most laboratories. Standard identification methods, including capsule synthesis, melanin production, urease production and ability to grow at 37 oC, could not differentiate between *C. neoformans* and *C. gattii*. Canavanine-Glycine Bromthymol blue agar (CGB) agar is usually not included in routine laboratory for *Cryptococcus* identification. However, the need for species identification is controversy as a recent guideline for treatment of cryptococcosis shows no different between *C. neoformans* and *C. gattii* treatment.

CrAg Screening: Pearls from the Pearl of Africa – Uganda

David R Boulware
University of Minnesota

David R Boulware will present lessons learned from CrAg screening and preemptive therapy from Uganda. The presentation will discuss: 1) successes and shortcomings in the current recommended preemptive therapy regimen of fluconazole monotherapy; 2) Simple methods of risk stratification to identify those at high risk of failing preemptive therapy; 3) Opportunities to improve outcomes of preemptive therapy with current and novel antifungal agents. There are many reasons to be happy with CrAg screening and preemptive therapy to improve survival of persons living with AIDS.

Cryptococcosis in Koalas

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Cryptococcosis is a devastating disease of koalas that is difficult to successfully treat.Understandably, it is caused predominantly by the *Cryptococcus gattii* species complex in this host because of the association between arboreal koalas and eucalypts.To date, all well documented free-livingkoalas with cryptococcosis and most captive koala cases have been caused by *C. gattii* VGI/AFLP4. Amongst captive koalas, there have also been a small number of cases caused by *C. gattii* VGII/AFLP6 in Australia. An evolving taxonomy of the *Cryptococcus neoformans- C.gattii* species complex over the past 30 years has meant that historical and sometimes even contemporary data can be difficult to interpret epidemiologically, aspathogenic *Cryptococcus spp*. are often identified in commercial veterinary diagnostic laboratories without molecular characterization. Anecdotal reports of *C.neoformans* associated disease (in contradistinction to *C. gattii*) in historical reports have never been verified using molecular methods or immunohistology because of the lack of isolate storage systems in busy commercial veterinary laboratories and limited archiving of paraffin-embedded formalin-fixed tissue specimens. Other *Cryptococcus* species have been isolated occasionally in association with koalas (mucosal surfaces and environment) around the world but none definitively associated with clinical disease. Koala cryptococcosis is a fascinating model of naturally-occurring disease in which the concept of a subclinical/asymptomatic state, paralleling human cryptococcosis, is well established. A clear link between the prevalence of clinical/subclinical cryptococcosis and environmental prevalence, enables molecular epidemiologic investigations to be employed both in captive and wild koala populations.Parallel investigations in artificial captive koala populations and wild koala populations is hoped to enable insights into the key microbiological features restricting an isolate to an environmental niche or potential progression to colonization or disease upon contact with the koala.Wild or captive koalas with subclinical cryptococcosis on the east coast of Australia (constituting the koala's natural range) are associated with VGI/AFLP4 isolates. A minority of cases are caused by VGII/AFLP6 isolates of the *C. gattii* species complex, and these have to date all had a physical connection to a point source in south Western Australia, some fourthousand kilometers away, after translocation of colonized animals. The impact of host factors affecting the host-pathogen-environment interaction remains difficult to assess with limited understanding of the normal koala immune system, unique behavioral and anatomical features and the impact of an endogenised retrovirus still uncertain. Young animals just achieving independence in highly contaminated environments do appear to be a particular group at risk for the development of clinical disease.Treatment of cryptococcosis in the koala kept in captivity is squarely aimed at identifying and managing subclinical disease, reducing the environmental burden of the potential pathogen, as management of advanced clinical disease is extremely difficult, requiring long term treatment using subcutaneous amphotericin infusions and or long courses of orally-administered azoles. Further challenges relate to the unique ability of this species to metabolise xenobiotics, which means therapeutic monitoring of drug levels is mandatory.

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Cryptococcus in wildlife species: fresh insights and their use as sentinels for human disease

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Cryptococcosis is a sporadic and uncommon fungal infection throughout the world, but is more common in certain geographical areas e.g. Australia, the Pacific North West of North America. The range of animals susceptible to infection is perhaps greater than for any other pathogen. It includes a wide diversity of wildlife, companion and production animals, and people. Other than koalas and companion animals, which are not the main focus for this session, clinical infections and/or asymptomatic carriage are reported in many terrestrial and aquatic placental mammals, marsupials, monotremes, birds, reptiles, amphibians and fishes. Most infections are associated with involvement of the upper respiratory tract, lungs and central nervous system (including eyes and optic nerves). Widespread dissemination leading to generalized organ and tissue involvement is also reported in a subset of animals (Psittaciformes, Ferrets), especially when disease is advanced and long-standing. The overall picture of cryptococcal disease in wildlife is complex. The clinical and diagnostic findings from wild animal populations are disparate, so the challenge is to develop a conceptual framework which permits the complexity to be understood in order that fresh insights can be determined. Indeed, published information is fragmentary, ranging from a single case due to opportunistic necropsy examinations after accidental death (e.g. foxes, elk) to more detailed case studies where patients live in a captive environment (i.e. zoo or Wildlife Park e.g. 'the koala story') or are involved in an outbreak event (e.g. Vancouver Island; goats in Spain). In addition, the accuracy of pathogen identification is influenced by the nature of the diagnostic specimens collected and by the availability of variably sophisticated laboratory tools (i.e. histopathology, cytology, culture, molecular methodologies). The *Cryptococcus neoformans* and *C. gattii* species complexes have been identified as the main important causes of infection in wildlife with higher prevalence in aquatic mammals and parrots from endemic areas (Western Canada, Australia and Brasil respectively), and especially in koalas which are arboreal marsupials which live in eucalyptus trees strongly associated with *C. gattii*. Additional epidemiologic information is provided by the monitoring of many wild and synantropic animal populations, usually performed in concert with other public health actions (e.g. avian influenza surveillance in birds and approved 'culling' of a overabundant species). In general, results from such studies suggest that wild animals are more likely to be asymptomatic carriers (i.e. subclinical disease) than clinically affected by cryptococcosis. Overall, migratory birds show lower prevalence of *C. neoformans* and *C. gattii* isolates compared to less virulent species such as *C. albidus* and *C. laurentii*. A caveat to these generalizations is that biochemical identification alone (e.g. API tests) can misidentify yeasts and produce erroneous diagnoses. Therefore, we suggest to routinely use modern molecular techniques to assess the genetic diversity of Cryptococci organisms in any epidemiologic or clinical setting in wild animals, ideally performed in a mycology reference laboratory or a lab with a special interest in *Cryptococcus.* We contend that surveillance of wildlife can provide an early warning system for outbreaks of new or emerging variant pathogen strains helping to inform an accurate risk assessment. Standardization of protocols and tools is needed to make a global comparison possible. Stated another way, wildlife species can be very important sentinels for the *Cryptococcus neoformans/gattii* species complexes, and provide new and penetrating insights into key epidemiologic and pathogenic mechanisms.

Cryptococcosis in domestic animals

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Cryptococcosis is a systemic mycosis caused by species of the *Cryptococococcus neoformans* complex and *Cryptococococcus gattii* complex,affecting human and animal hosts. It is the most common systemic mycosis of cats, but other domestic mammals may also be infected, including dogs, horses, goats, sheep and cattle. Moreover,other species of the genus Cryptococcus, such as *C. albidus* (horses, cats, and dogs), *C. magnus* (cats), *C. laurentii* (dog), and *C. flavescens* (dog), have occasionally been reported as etiologic agents of fungal infections.Feline cryptococcosis occurs worldwide, being most frequently reported in Australia, Canada and the United States.The natural route of infection is usually through inhalation. The most common sites of infection are the nasal cavity, skin, lymph nodes, CNS and eyes. It is not considered a contagious or anthropozoonotic disease, representing a fungal infection acquired from the environment, with animals serving as potential sentinel hosts for human exposure. However, some cases of zoonotic transmission of *C. neoformans* from birds raised as pets have been described. In addition, the importance of human cryptococcosis as an infection commonly associated with AIDS patients must be emphasized. The emergence of this mycosis and geographic dispersal of their agents in the world is reinforced by the significant increase in scientific production on this issue. An update on animal cryptococcosis will be presented.

Regulation of ubiquitin-proteasome in *Cryptococcus* pathogenesis

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Cryptococcus neoformans is a facultative intracellular fungal pathogen that infects the lung and then often disseminates to the central nervous system to cause meningitis. Alveolar macrophages are the first line of host defense against *Cryptococcus* infection. How *Cryptococcus* is able to suppress host immunity and escape the antifungal activity of macrophages remains incompletely understood.We recently reported that the F-box protein Fbp1, a subunit of the SCF^{Fbp1} E3ligase, promotes *Cryptococcus* virulence by regulating the fungal proliferation inside macrophages. We showed that the role of Fbp1 in fungal infection is independent of known classical virulence factors (capsule, melanin, and growth at 37C), suggesting that the SCF^{Fbp1} E3 ligase regulates a novel virulence control mechanism. Interestingly, the *fbp1* mutant elicited superior protective Th1 host immunity in the lung as compared to the wild-type strain, suggesting that Fbp1functions as part of a mechanism leading to immune suppression. Moreover, *fbp1* yeast cells conferred protection against a subsequent infection with the virulent H99 parental strain, indicating that the *fbp1*Δ strainmaybe used in vaccination strategies. Altogether our findings suggest that inactivation of Fbp1 allows the host to develop a robust immune response to *C. neoformans* even in the presence of other dominant virulence factors. Besides its critical role in fungal virulence, Fbp1 also controls fungal sporulation. Because E3 ligases regulate cellular activities by controlling the turnover of their substrates, we sought to identify Fbp1 substrates required for Fbp1-mediated pathogenicity and sporulation by employing a combination of genetic and proteomics approaches. We discovered that inositol phosphosphingolipid phospholipase C1 (Isc1) is an Fbp1 substrate involved in *Cryptococcus* intracellular growth inmacrophages. We are currently investigating the Fbp1-mediated regulation of sphingolipid biosynthesis and its role in *Cryptococcus* virulence. We also identified CDK-related kinase Crk1 as an Fbp1 substrate which regulates fungal sporulation and virulence. The long-term goal of this project is to identify and characterize Fbp1-regulated fungal effectors and corresponding host immune factors that determine disease progression to understand the host-pathogen interaction during pulmonary infection.

Regulated secretion of the immunomodulatory polysaccharide GXM facilitates cryptococcal dissemination

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The basidiomycetes yeast *Cryptococcus* is the most prevalent cause of fungal meningitis, responsible for~1 million diagnosed cases and ~600,000 deaths annually. Cryptococcal infection begins in the lungs after the inhalation of infectious particles. In immunocompromised individuals, where symptomatic disease is most prevalent,fungal cells disseminate from the lungs and exhibit a particular tropism for the brain, leading to cryptococcal meningitis.Cryptococcal meningitis patients often suffer high titers of infecting fungal cells within the brain,but exhibit limited neuro-inflammation. In mouse models of disseminated cryptococcosis, immune cell infiltration into the central nervous system (CNS) is also extremely limited. When we infect C57Bl/6NJ mice intranasally with *C. neoformans*, we detected little immune cell infiltration (macrophages, neutrophils, CD4+, and CD8+ cells) into infected brains, despite high fungal burden and histological damage.We also detected many small deposits (<5 m) of the polysaccharide glucuronoxylomannan (GXM) in brain and spinal cord tissue of infected mice.*Cryptococcus* species produce a polysaccharide capsule, consisting primarily of GXM, that is necessary for virulence. GXM is also found free, unattached to fungal cells, in the serum and cerebrospinal fluid (CSF) of infected patients.Since GXM has well-characterized immunosuppressive properties, we hypothesized that this free GXM (exo-GXM) is responsible for the lack of CNS inflammation.To test this, we infected mice intracranially with an a capsular strain (cap60Δ).We found that the brain-infiltrating immune cells significantly increased compared to mice infected with wild-type fungi. Administration of purified GXM by intraperitoneal injectionto cap60Δ-infected mice suppressed immune cell infiltration into the brain and increased fungal burden. We have also identified mutants deficient in exo-GXM production and the regulation of the switch between cell surface retention and exo-GXM secretion.Mutations that alter exo-GXM levels but not cell surface capsule levels also alter immune cell infiltration into the brain. Our results support a model in which *C. neoformans* actively secretes exo-GXM to modulate the host's immune responses and promote fungal survival in the brain

The dynamics of the *Cryptococcus neoformans* transcriptome

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Our recent work suggests that a fascinating, complex pattern of RNA molecules composes the *Cryptococcus* transcriptome and this fungus is emerging for different aspects as an ideal model to study RNA metabolism in eukaryotes. We have recently re-annotated the genomes of both varieties of *C. neoformans* and proved that introns represent key players in the regulation of gene expression in this yeast. More recently, we identified a large diversity of transcripts in *C. neoformans*. In addition to transcripts resulting from alternative splicing, a genome wide analysis of transcription start sites using TSS-Seq and polyadenylation sites using 3UTR-Seq revealed that a large number of transcripts are resulting from alternative starts sites or alternative polyadenylation sites. The consequences of the proteome diversity will be discussed. Nevertheless, it is tempting to hypothesis that this complex RNA metabolism provides a mechanism for this yeast to respond to different environmental cues and to be an efficient pathogen.

Leading the charge – South Africa's evolving public health response to cryptococcosis over 15 years

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South Africa (SA) has the world's largest generalised HIV epidemic (˜6.7 million persons) and has implemented a rapidly-expanding antiretroviral treatment (ART) programme to match this (˜3.3 million persons). Despite these efforts, SA still falls short of the ambitious UNAIDS 90-90-90 targets: only 76% of those living with HIV know their status, only 65% of those who know their status are on ART and of these, 84% have virologic suppression. These gaps in the HIV cascade of care partly explain why advanced HIV disease and cryptococcosis in particular remains a major public health problem in 2017. The burden of cryptococcal meningitis, documented by laboratory-based surveillance, has remained persistently high in SA over the last 15 years.HIV/AIDS-related deaths account for almost a third of all deaths in the SA population and cryptococcal meningitis, whichin turn has been estimated to be responsible for 15% of AIDS-related mortality, probably contributes substantially to this total. In 2011, the World Health Organization recommended that countries consider integrating cryptococcal antigen (CrAg) screening into HIV programmes to detect cryptococcal disease at an earlier point in its trajectory and thus reduce AIDS deaths by pre-emptive antifungal treatment. In 2012, this intervention was included in SA's National Strategic Plan for HIV/AIDS, tuberculosis and STIs. Since then, evidence has mounted in favour of the CrAg screen-and-treat intervention. On 1 November 2016, SA's National Health Laboratory Service (NHLS) implemented the world's largest national laboratory CrAg screening programme. Linked to baseline CD4 count testing of patients entering (or re-entering) the ART programme, a projected 250 000 HIV-infected patients with a CD4+ T-lymphocyte count below 100 cells/µl will be screened for CrAg annually. This was the culmination of a nationally-coordinated effort over many years by a large group led by the National Institute for Communicable Diseases (NICD), SA's Department of Health and partners. In this talk, I will review key activities preceding this milestone such as piloting implementation and large-scale field evaluations of reflex and provider-initiated laboratory CrAg screening approaches in parallel, development of a detailed cost-effectiveness model comparing these approaches, laboratory evaluation of CrAg assays, conducting a public health planning and resource allocation exercise and development of a real-time national "monitoring dashboard" for use at different levels of the health system. I will also discuss future directions for the CrAg screen-and-treat intervention in SA and an increased overall programmatic focus on persons with advanced HIV disease.

Trends in cryptococcal incidence in Botswana

Joe Jarvis
Johns Hopkins University

Botswana has one of the best antiretroviral therapy (ART) programme in Africa, with reported ART coverage close to the UNAIDS 90-90-90 targets. With wide availability of ART, the burden of advanced HIV disease and associated opportunistic infections such as cryptococcal meningitis would be expected to decline. We performed a nationwide surveillance study determining the national incidence of cryptococcal meningitis, and describing the characteristics of cases from 2000-2015 and temporal trends at the two national referral hospitals.5296 episodes of cryptococcal meningitis were observed in 4702 individuals during the study period; 90.3% (4248/4702) had a single episode, 60.6% (2670/4407) were male, and median age was 36 years. Overall 2013-2014 incidence was 17.8 cases / 100,000 person-years (95%CI 16.6 – 19.2 cases / 100,000 person-years) with peak incidence in males aged 40-44 years. In the HIV-infected population, incidence was 96.8 cases / 100,000 person-years (95%CI 90.0 – 104.0 cases / 100,000 person-years). Case numbers decreased from 2007-2009 but stabilised from 2010-2014. Despite excellent ART coverage in Botswana, there is still a substantial burden of advanced HIV disease. We observed high ongoing incidence of cryptococcal meningitis comparable to pre-ART era incidence rates in South Africa. Our findings suggest that a key population of individuals, often men, are developing advanced immune suppression and associated opportunistic infections due to a failure to effectively engage with HIV testing and treatment services, highlighting the need for differentiated models of care.

Cryptococcus is talking……. Are we listening??

John R. Perfect, MD
Duke University Medical Letter

As we approach 125 year since the first human case report of *cryptococcus* causing disease, what do we know and what do we need to know? It is important to note that molecular biological tools and genomics to help understand this encapsulated pathogen have been unleashed on this disease. With the use of molecular techniques and robust animal models, we have begun to create an understanding of how this pathogen produces disease and how the host responds to it. Cryptococcosis clearly lives in the Goldilock's paradigm of immunity…. "Not too much and not too little…. The host needs to get it just right!!!" In this discussion, there will be a merger of molecular epidemiology through whole genome sequencing to frame the relationships between the *Cryptococcus* complex (strain vs species; environmental vs clinical isolates). How have yeast investigations directly from the host (RNA seq on the yeast at the site of infection) allowed the yeast to tell us what it is thinking and how do genetic changes of the yeasts in the host identify its weak points for exploitation? We can start to review the results and make interpretations. Furthermore, from epidemiological standpoint what is happening with the cryptococcal outbreak in 2016 and are there changes in risk groups occurring even today? Maybe Management is always a dynamic issue and yet no new anti-cryptococcal drugs have been approved for use in the last 2 decades. What are our failures? How can or cannot the Cryptococcal Guidelines help us? Do we use our diagnostics better and how should we? Have we become a split world between resource-available vs resource-limited area in our management of cryptococcal meningitis? Of course, the answer is "yes". How do we bring these groups together or should we? All these questions will require more investigation but in this presentation we will try to specifically exam: (1)The use of CRAG for pre-emptive therapy;(2)Attempt to frame the clinical issues around *C. neoformans* vs *C. gattii* disease; (3) Guideline updates (lipid products of amphotericin B for all and the use of quantitative CSF yeast counts in management);(4) New drugs coming but how soon and can creative strategies like Neurapheresis be on our clinical door step soon? What makes sense to improve our management? Crytococcosis is not going away anytime soon but can we do a better job with it today as we wait for the understandings of tomorrow. Many, many questions……..Let's provide a few answers!

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ABSTRACTS

ORAL PRESENTATIONS

Rewiring of signaling networks modulating thermotolerance in the human pathogen *Cryptococcus neoformans*

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Thermotolerance is a crucial virulence attribute for human pathogens, including the fungus *Cryptococcus neoformans* that causes fatal meningitis in humans. Loss of the protein kinase Sch9 increases *C. neoformans* thermotolerance, but its regulatory mechanism has remained unknown. Here, we studied the Sch9-dependent and Sch9-independent signaling networks modulating *C. neoformans* thermotolerance by using genome-wide transcriptome analysis and reverse genetic approaches. During temperature upshift, genes encoding for molecular chaperones and heat shock proteins were upregulated, whereas those for translation, transcription, and sterol biosynthesis were highly suppressed. In this process, Sch9 regulated basal expression levels or induced/repressed expression levels of some temperature-responsive genes, including heat shock transcription factor (HSF1) and heat shock proteins (HSP104 and SSA1). Notably, we found that the HSF1 transcript abundance decreased but the Hsf1 protein became transiently phosphorylated during temperature upshift. Nevertheless, Hsf1 is essential for growth and its overexpression promoted *C. neoformans* thermotolerance. Transcriptome analysis using an HSF1 overexpressing strain revealed a dual role of Hsf1 in the oxidative stress response and thermotolerance. Chromatin immunoprecipitation demonstrated that Hsf1 binds to the step-type like heat shock element (HSE) of its target genes more efficiently than to the perfect- or gap-type HSE. This study provides insight into the thermotolerance of *C. neoformans* by elucidating the regulatory mechanisms of Sch9 and Hsf1 through the genome-scale identification of temperature-dependent genes

Neuro-Immune Responses during Cryptococcal Meningitis

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Cryptococcus neoformans infections are a significant cause of morbidity and mortality among AIDS patients and the third most common invasive fungal infection in organ transplant recipients. Immunocompromised patients are the most susceptible to cryptococcosis, highlighting the vital role of host immune responses in control of this infection. *C. neoformans* is generally acquired via inhalation and the pulmonary immune response to this pathogen has been extensively studied. However, most patients present with meningitis, due to the ability of this neurotropic pathogen to invade the central nervous system (CNS). Despite this, little is known about how *C. neoformans* invades the CNS or the resulting neuroimmune responses, which remain significant gaps in our understanding of cryptococcal pathogenesis. To investigate these questions, investigators have employed various techniques, including histopathology and intravital microscopy, typically after injecting high numbers of organisms directly into the blood stream to efficiently seed the CNS. We are using a far-red florescent *C. neoformans* strain to characterize the neuro-immune response during infection by flow cytometry. Because *C. neoformans* is generally acquired via inhalation, we chose to compare the intravenous (I.V.) route of infection, typically used in earlier studies, to the more physiological intranasal (I.N.) route of infection. On day 12 post-inoculation, mice infected I.V. had significantly more infiltrating immune cells in the brain compared to mice infected with the same inoculum intranasally. The I.V. infected mice also had much higher fungal burden in the brain compared to the intranasally infected mice. To test whether the increase in immune infiltrates was due to higher fungal burden, we titered the amount of *C. neoformans* infected I.V. to achieve comparable fungal burdens in the I.V. and I.N. infected mice. Nonetheless, the I.V. infected mice still had greater levels of immune cell infiltration. Interestingly, regardless of the route of infection, most of the organism was associated with microglia. Altogether, our studies suggest that studies of the neuro-immune response to *C. neoformans* must carefully consider the route of infection

Cryptococcus gattii and Influenza A: Together become worse

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Cryptococcus gattii is one of the main etiologic agents of cryptococcosis, a disease that affects lungs and the central nervous system (CNS), causing meningoencephalitis in immunocompetent individuals. Occasionally, the pathophysiology of cryptococcosis may be influenced by other pathogens in a coinfection situation; among them, one case report the coinfection with Cryptococcus and influenza A virus. The influenza A infections are a major public health concern and cause annual epidemics with significant morbidity and mortality worldwide. Considering that (i) both pathogens affect the respiratory tract and the (ii) relevance and severity of infections caused by influenza A and by Cryptococcus spp., this study aimed to outline an in vivo model of coinfection with C. gattii and influenza A and evaluate the influence of the virus in the progression of cryptococcosis. For this, C57BL/6 mice were divided in the following groups: 1) infected with influenza A H1N1 intranasally; 2) infected with C. gattii intratracheally and 3) infected with C. gattii after viral inoculation. We verified that mice infected with both pathogens presented increased morbidity ($p<0.05$) and early mortality (death until 8 days) compared to animals infected only with C. gattii (death until 30 days); while animals infected only with the virus did not succumb even after 100 days post infection. The antiviral treatment with oseltamivir increased the survival of coinfected mice; in contrast, this result was not observed when coinfected mice were treated with fluconazole. Furthermore, influenza A and C. gattii coinfection, lead to a significant increase ($p<0.05$) in the fungal load in the CNS and in the recruitment of neutrophils and lymphocytes to the bronchoalveolar lavage fluid. Macrophages previously infected with the virus have decreased ability to engulf and kill the fungus. The inefficient antifungal activity of macrophages was associated with the decreased levels of INF-γ (a key cytokine for the macrophage activation) in the lungs, which was a probable consequence of the increased expression of INF-α and INF-β in this organ. These results suggest the negative influence of the influenza A virus in cryptococcosis. In addition, this work provides knowledge of the interaction between these infectious agents and host during coinfection.

CCR2+ inflammatory monocytes are a regulatory checkpoint for the pulmonary immune response to *Cryptococcus neoformans* challenge

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The role of CCR2+ Ly6Chi inflammatory monocytes (Mo) in the immune response to *Cryptococcus neoformans* (Cn) infection is not yet clearly defined. Using a respiratory infection model of cryptococcosis,we find that transient ablation of CCR2+ Mo in CCR2-DTR mice improves survival and fungal burden in the lung, mediastinal lymph nodes and brain compared to C57BL/6 wild type (WT) littermates after infection with Cn strain H99. Lung levels of IFN-gamma, TNF-alpha,IL-12, IL-17, IL-5, and RANTES/CCL5 are decreased in CCR2-DTR mice compared to WT mice on day 7 post-infection (p.i), consistent with the idea that depletion of CCR2+ Mo suppresses damaging inflammatory responses. In CCR2-DTR lungs, we observe a significant increase in neutrophils on day 14 p.i. and a decrease in eosinophils on day 7 and 14 p.i. compared to WT lungs. These data suggest that CCR2+ Mo promote eosinophil recruitment through the regulation of IL-5 and RANTES/CCL5. However, this pulmonary eosinophilia is not essential to disease progression since eosinophil-deficient B6-GATA mice have similar survival and lung fungal burden as WT mice. We also find that the regulatory role of CCR2+ Mo in the pulmonary immune response to cryptococcosis may depend, in part, on intrinsic signaling through spleen tyrosine kinase (Syk). Initial studies using novel CCR2-Cre mice show that conditional knockout of Syk in CCR2+ Mo worsens survival compared to control mice. These results support a novel pathway in which Cn induces CCR2+ Mo to promote fungal proliferation and disease progression through the modulation of cytokine production and cellular crosstalk with granulocytes. Infectious outcomes can be improved by ablation of CCR2+ Mo or preservation of CCR2+ Mo-intrinsic Syk signaling. Further investigation of these cellular and molecular mechanisms may identify new immunomodulatory targets for anti-cryptococcal therapy

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Prevalence of latent cryptococcosis among HIV-infected patients in Cameroon: the ANRS 12312 PreCASA study

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Cryptococcal meningitis is a leading cause of mortality among adults with HIV/AIDS in Africa. Cryptococcosis is one of the few infectious diseases whereby cryptococcal antigen (CrAg) can be detected in plasma weeks to months prior to the onset of symptoms. Consequently, to decrease CM-associated mortality, the World Health Organization (WHO) recommends the consideration of routine screening for latent cryptococcosis and pre-emptive fluconazole therapy in antiretroviral therapy (ART)-naïve patients who present to HIV programs with <100 CD4 cells/mm3, especially in areas where CrAg prevalence is ≥ 3%. However, in Central Africa, there has been no data to date on the prevalence of latent cryptococcosis (i.e. CrAg antigenemia). In an ongoing cohort study, using the IMMY® point of care CrAg lateral flow assay (LFA), we consecutively screened for CrAg in all asymptomatic consenting adult ART naïve patients who presented to the Day Hospital of Yaoundé Central Hospital in Cameroon with <100 CD4 cells/mm3. Study participants who were found to be CrAg positive in plasma were consented for a lumbar puncture to collect cerebrospinal fluid (CSF) for India Ink staining and fungal culture to ascertain or not whether they had cryptococcal meningitis. Those who were CrAg positive with no microbiological evidence of cryptococcal meningitis were immediately placed on pre-emptive fluconazole-based therapy, and ART deferred by 2 weeks. CrAg negative patients were immediately placed on ART and all enrolled participants followed up for one year to determine the incidence of CM during the first year of ART. In the study population of 164 adults enrolled between July 2015 and October 2016, 70.7% were women. The mean age was 38.1 years (Standard deviation [SD]:10.1). The median CD4 cell count was 46 cells/mm3 (Interquartile range [IQR]: 27 – 79), with men having lower median CD4 cell counts than women (40 [IQR: 21 – 66] vs 49 [IQR: 28 – 81], p=0.04). The prevalence of CrAg in plasma in the study population was 8.5% (95% Confidence Interval [CI]: 5.1 – 13.9), 12.5% in men and 6.9% in women (p=0.4) and 5.9% and 11.8% in those with <50 CD4 and >50 CD4 cell counts, respectively (p=0.3). Among the 14 CrAg positives, 5 (35.7%) had culture proven cryptococcal meningitis in CSF, with a median CD4 count of 38 (IQR: 29 – 55, p=0.12). All patients with culture proven cryptococcal meningitis were treated with recommended regimen in an ongoing clinical trial (ACTA trial). In conclusion, cryptococcosis is endemic among severely immune depressed HIV patients in Cameroon. Sub-clinical cryptococcal meningitis is common among CrAg positive patients and occurred in just over a third of patients. As such, routine screening for CrAg and pre-emptive fluconazole-based treatment should be implemented in Cameroon prior to initiating ART and form part of policy; in addition to ongoing efforts to implement optimal management of patients presenting with symptomatic cryptococcal meningitis.

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HDAC genes play distinct and overlapping roles in *Cryptococcus neoformans* virulence

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Chromatin remodeling is involved in several cell processes such as stress response, adaptation, immune response and carcinogenesis. However, chromatin remodeling mechanisms are poorly understood in pathogenic microorganisms, particularly in the context of virulence phenotypes. The ability to adapt to environment changes and/or to interact with host cells ensures the survival and proliferation of different pathogens. The human fungal pathogen *Cryptococcus neoformans*, na encapsulated yeast, undergoes many phenotypic changes to promote proliferation and survival in specific ecological niches and also inside the host organism. This phenotypic plasticity occurs in response to different environment signals and is likely related to chromatin remodeling. Histone deacetylase (HDAC) genes are highly conserved among different fungal species, and the enzymes they encode coordinate major processes in chromatin dynamics. Previously, we evaluated the effect of two chemical inhibitors of the histone deacetylase activity [HDACi; sodium butyrate (NaBut) and Trichostatin A (TSA)] on the expression of the main virulence phenotypes in *C. neoformans*. Our results showed that both drugs were able to impair the expression of the main virulence traits of this fungus. In the current work, we identified and deleted the eight genes encoding predicted class I/II HDAC in the *C. neoformans* genome. Therefore, we could assign specific functions to each HDAC, particularly concerning virulence trait expression. Our results indicate that individual enzymes control non-identical but overlapping cellular processes associated with virulence. These processes include thermotolerance, capsule formation, melanin synthesis, protease activity and cell wall integrity. We also determined the HDAC genes necessary for *C. neoformans* survival during in vitro macrophages infection and in animal models of cryptococcal infection. Our results identified a new HDAC key gene, CLR3, as pivotal for the control of several virulence processes. CLR3 gene deletion resulted in impaired mating hyphae formation and pronounced hypovirulence in invertebrate and mouse infection models. Finally, a global gene expression profile performed by RNAseq, comparing the clr3Δ versus the wild-type strain, showed significant reduction in the transcription of several genes associated with the most prominent virulence attributes. Our results clearly demonstrate that chromatin remodeling is crucial to virulence control in *C. neoformans*. This is the first study that directly correlates phenotype plasticity /virulence to chromatin remodeling in this microorganism. We believe our data can contribute to the design of new therapeutic approaches for the treatment of cryptococcosis.

Potent Activity of VT-1598 Alone or in Combination with AmBisome in a Murine Model of Cryptococcal Meningitis

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Cryptococcal meningitis (CM) continues to be a major cause of morbidity and mortality in immunocompromised patients, with >200,000 deaths per year. Treatment outcomes remain poor, with mortality rates of 20-30% despite access to the best available treatment regimens and specialized medical care, hence more effective and safer drug regimens are urgently needed. We report here the potent, fungicidal-like activity of VT-1598 monotherapy in a murine model of CM, and increased antifungal activity in the presence of amphotericin B. *In vivo* antifungal activity was determined in a tail-vein model of CM in immunocompetent mice, inoculated with *C. neoformans* H99 (N=10 per dose group). A 6-d treatment started 1-d post-inoculation, followed by a 1-d wash-out, with brain fungal burden and plasma compound levels measured at the end of study. The first study examined VT-1598 monotherapy at oral doses of 5, 15, 50 mg/kg once-daily; 10 mg/kg IV AmBisome once-daily; and 25 mg/kg oral fluconazole twice-daily. The second study examined monotherapy and combination treatments: 2.5 and 5 mg/kg oral VT-1598 once-daily, 7.5 mg/kg IV AmBisome once-daily, and the combinations of 2.5 mg/kg VT-1598/7.5 mg/kg AmBisome and 5 mg/kg VT-1598/7.5 mg/kg AmBisome, as well as 25 mg/kg oral fluconazole twice-daily.In the monotherapy study, VT-1598 treatment resulted in potent dose-dependent decreases in brain fungal burden. Furthermore, the burdens in the mid and high doses were 8- and 27-fold lower than the *input burden* at the time of treatment initiation, suggesting fungicidal-like activity. At the start of treatment, the mean burden was 3.25 log10 CFU/g, which increased to 7.74 log10 in the vehicle group at the end of the study. The burdens in the low, mid, and high VT-1598 dose groups were 3.39, 2.36, and 1.82 log10, respectively, with corresponding VT-1598 plasma levels of 2.2, 6.7, and 17 g/ml. The positive comparators yielded reductions within the expected range of the model (3.16 and 3.86 log10 for AmBisome and fluconazole, respectively). Fungal burden reductions in both the mid and high VT-1598 doses were statistically superior to both comparator drugs (P <0.0001). In the combination treatment study, vehicle control fungal burden (7.93 log10) was similar to the first study. The expected fungal burden reductions were observed in each monotherapy group (2.5 mg/kg VT-1598 = -2.47 log10; 5 mg/kg VT-1598 = -4.56 log10; 7.5 mg/kg AmBisome = -3.61 log10). In comparison, the combination treatments increased the antifungal reductions by 4.8 – 42-fold (P <0.0001). As one example, the combination of 2.5 mg/kg VT-1598/7.5 mg/kg AmBisome where both drugs were measurably below optimal plasma levels (0.51 – 0.78 g/ml), the fungal burden was 3.31 log10 (corresponding to a 4.6 log10 reduction compared to vehicle control). These preclinical studies demonstrate that VT-1598 has potent antifungal activity in a murine model of CM as monotherapy and in combination with AmBisome. As such, these data support clinical studies of both monotherapy and combination treatments in patients with this devastating disease.

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Molecular biomarkers of paradoxical Cryptococcosis-associated immune reconstitution inflammatory syndrome

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Paradoxical Cryptococcosis-associated immune reconstitution inflammatory syndrome (C-IRIS) occurs in ~25% of HIV-infected patients with cryptococcal meningitis (CM) after they commence antiretroviral therapy (ART). The pathogenesis of C-IRIS remains poorly understood, with few biomarkers to predict C-IRIS risk or to establish an early diagnosis. We investigated whole blood transcriptomic profiles of 54 ART-naive, HIV-infected participants with confirmed CM, nested in the Cryptococcal Immune Restoration Disease study (Chang AIDS 2013). 27 patients with probable or possible C-IRIS and 27 participants (no C-IRIS) were matched by CD4+ T cell counts at baseline. Whole blood was collected into PAXgene tubes at the time of ART initiation, after 4 and 12 weeks, and at the first symptoms of neurological deterioration associated with CIRIS. For each blood sample, total RNA was extracted and analyzed using Illumina HT-12v4 microarrays. Hybridization intensities were normalized by the quintile normalization method using Genome Studio software, and statistical analyses were performed using Partek Genomic Suite v7. Differentially expressed genes were mapped using Ingenuity Pathway Analysis software. At the time of ART initiation, we found no transcriptomic differences between patients with C-IRIS and those without C-IRIS. Longitudinal analyses of transcriptomic profiles in those that did not develop C-IRIS at weeks 4 and 12 after initiation of ART identified 250 and 280 transcripts that had changed expression compared to their pre-ART expression. Transcripts that were down-regulated following ART initiation encoded components of antiviral pathways, including interferon signaling pathways and apoptosis pathways, that we previously identified to be involved in HIV pathogenesis (JAIDS 55:428-38, 2010). Comparison of transcriptomic profiles in those who did or did not develop C-IRIS allowed us to identify approximately 600 transcripts that were differentially expressed at the time of C-IRIS events. Based on transcriptomic signatures, we separated C-IRIS events into two distinct subclasses: early C-IRIS events (occurred within 4 weeks on ART), and late C-IRIS (occurred after 12 weeks on ART). Interesting features of early-C-IRIS events included upregulation of transcripts encoding pattern recognition receptors and inflammasome components, as compared to corresponding baselines. Late C-IRIS event were characterized by abnormal overexpression of transcripts encoding pro-inflammatory cytokines and receptors expressed on T-, B- and NK-cells. These results suggest that an alteration in the balance between innate and adaptive immunity is associated with C-IRIS events. Thus, we have identified a unique transcriptomic signature, which discriminates the CIRIS subgroups from those undergoing appropriate immune restoration (no C-IRIS). This information provides insight into the pathogenesis of C-IRIS that could be applied to developing diagnostic tests or targeted immunomodulatory treatments.

The Human Blood-Brain Barrier Internalizes *Cryptococcus neoformans* via the EphA2-Tyrosine Kinase Receptor

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Fungal infections in the central nervous system (CNS) cause significant morbidity and mortality. *C. neoformans* can move freely through the blood-stream and crosses the blood-brain barrier (BBB) endothelium via a transcytosis mechanism. This is an incredible achievement given that the BBB exists largely to protect the CNS by restricting entry of undesirable bloodborne molecules and potentially threatening agents. Here we resolved the transcriptome of the human brain microvascular endothelial cells (hBMECs, a.k.a. BBB) challenged with C.neoformans in order to establish whether *C. neoformans* invaded the CNS by co-opting particular signaling pathways as a means to promote its own entry. The transcriptome was mapped to known canonical signaling pathways and ratios of differentially expressed genes to the total number of genes attributed to each pathway were determined. We found that when the BBB is challenged with *C. neoformans*, 5 major canonical signaling pathways are activated and play key roles during the internalization of *C. neoformans*. The pathways identified were: 1) EPH-ephrin (EphA2) receptor tyrosine kinase pathway, 2) axonal guidance pathway involving MAPK signaling and cytoskeleton regulation, 3) RhoGDI (Rho GDP-dissociation inhibitors - regulators of Rho-GTPases in cytoskeleton-remodeling, vesicular trafficking and gene expression), 4) CXCR4-pathway (immune/inflammatory response), and 5) IL-8 signaling pathway (cell proliferation, survival and invasion). Interestingly, the EPH-ephrin (EphA2) receptor kinase-signaling pathway dominated the transcriptome; this pathway regulates several key processes including re-modeling of intercellular junctions, cytoskeleton reorganization and cell adhesion. We found that the EphA2 tyrosine kinase receptor is a primary target of *C. neoformans*. Silencing the EphA2 transcript in hBMECs or blocking EphA2 activity with an antibody or chemical inhibitor prevented transmigration of *C. neoformans* in an in vitro model of the BBB. In contrast, treating hBMECs with an EphA2 chemical agonist or an EphA2 ligand promoted greater migration of fungal cells across the BBB. C. neoformans activated the EPH-tyrosine kinase pathway through the phosphorylation of EphA2 – this promoted clustering and internalization of EphA2 receptors that to co-localized with F-actin and cryptococci. Collectively, the results suggest that *C. neoformans* activates EphA2 and this is turn creates a more permeable barrier that facilitates crossing of the BBB. These findings are far-reaching and particularly intriguing because of the known role of EphA2 in remodeling intercellular junctions – thus the *C. neoformans*-induced transcriptional changes in the BBB and a concomitant sustained activity of EphA2 could open a paracellular pathway and thus boost further entry of *C. neoformans* along with excess fluid that would lead to brain edema, a defining feature of meningoencephalitis. EphA2 might function to couple early-transcellular crossing with na eventual paracellular opening. Another intriguing aspect of this study is the notion that EphA2 might serve as a potential target for small molecules or drugs that could be used to prevent fungal meningoencephalitis. Given the use of the lateral flow assay for Cryptococcal antigen detection in under 10 min in a symptom-free HIV+ population, drugs given to prevent fungal crossing into the brain could significantly reduce cryptococcal meningoencephalitis-related deaths.

Identification of spore germination mutants in *Cryptococcus* using a counter-selective genetic screen of Agrobacterium-derived insertional transformants

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Cryptococcus neoformans is a fungal pathogen that causes lethal meningoencephalitis. Disease occurs when *Cryptococcus* is inhaled and disseminates to other tissues. Spores, products of both same- and opposite-sex development, are infectious particles capable of causing disease in animal models. Germination is the process by which spores transition into vegetatively growing yeast and is required for *C.neoformans* spore-mediated infections to cause disease. We hypothesize that there are genes and pathways specific to and/or required for germination. To test this hypothesis, we carried out a counter selectionbased screen to identify mutants that produce spores incapable of germinating into yeast. Using Agrobacterium-mediated transformation, we generated four pools of transformants (3,696 total transformants) and screened them for the ability to form germinating spores. We identified 23 transformants that produced spores that were unable to germinate and form colonies. Secondary screening of these hits will identify bona fide germination mutants. Further characterization of the mutations that result in defects during germination will provide new insights into pathways and genes that control the critical process of spore differentiation into yeast

Spore Germination as a Target for Antifungal Therapeutics

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Spores are critical cell types required for long-term survival of most fungi.The ability of spores to germinate and grow is essential for establishing colonies in new environments. For pathogens like *Cryptococcus*,germination is the key differention process required for spores to initiate vegetative growth and ultimately cause disease. Because germination is required for pathogenesis, identifying novel compounds that inhibit germination could provide opportunities for new therapeutics. Such compounds could be developed into low-toxicity drugs that could be used in the prevention and/or treatment of cryptococcosis (and other fungal sporemediated diseases).Using a novel luciferase-based high throughput assay, we screened a library of ~1200 FDA-approved drugs and identified several germination inhibitors and yeast growth inhibitors. These drugs have been approved for use in treating a wide variety of conditions not related to fungal disease, but they could be repurposed for use as antigermination and/or antifungal drugs. To assess the possibility of repurposing these drugs for use in prophylaxis and/or treatment of fungal disease, we determined the minimum inhibitory concentration (MIC) and the minimum fungicidal concentrations (MFC) of these drugs. In addition, using a recently developed, quantitative microfluidics-based germination assay, we determined the specific effects of compounds on germination with respect to stage of inhibition and kinetics.The pathways targeted by compounds of interest are currently under investigation through a variety of biochemical and molecular approaches.The identification of molecular mechanisms of germination inhibition will also help identify pathways with the potential for use as targets in future antifungal drug development