Closely related *Cryptococcus neoformans* strains possess differential virulence both in humans and the mouse inhalation model

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Abstract

Cryptococcal meningitis (CM) causes high rates of HIV-related mortality, yet *Cryptococcus* factors influencing patient outcome are not well understood. Pathogen-specific traits, such as the strain genotype and degree of antigen shedding, are associated with clinical outcome but the underlying biology remains elusive. In this study, we examined factors determining disease outcome in HIV-infected cryptococcal meningitis patients infected with *C. neoformans* strains with the same multi-locus sequence type. Both patient mortality and survival were observed during infections with the same sequence type. Disease outcome did not correlate with underlying patient immune deficiencies. Patient mortality was associated with higher antigen levels, fungal burden in the CSF, and low CSF fungal clearance. Virulence of a subset of clinical strains with the same sequence type were analyzed using the mouse inhalation model of cryptococcosis. We showed a strong correlation between human and mouse mortality rates, demonstrating the mouse inhalation model recapitulates human infection. Similar to human infection, the ability to multiply *in vivo*, demonstrated by high fungal burden in the lung and brain tissues, was associated with mouse mortality. Mortality rate was not associated with single *C. neoformans* virulence factors *in vitro* or *in vivo*; rather, a trend in mortality rate correlated with a suite of traits. These observations show that genotype similarities between *C. neoformans* strains do not necessarily translate into similar virulence either in the mouse model or in human patients. In addition, our results show that *in vitro* assays do not fully reproduce *in vivo* conditions that influence *C. neoformans* virulence.

Introduction

Cryptococcus neoformans is a fungal pathogen that causes disease mainly in immunocompromised patients such as individuals living with HIV/AIDS or receiving organ transplants. The availability of antiretroviral therapy has reduced AIDS-related mortality, however deaths due to cryptococcal meningitis (CM) have plateaued with *C. neoformans* still causing 15 % of all AIDS-related deaths globally (1, 2). Mortality rates due to *Cryptococcus* infection varies by region from 70% in low-income countries to 20-40% of all infected patients in high-income countries (2). Although differences in mortality rates between high- and low-income countries can be linked to sub-optimal antifungal treatments in low-income countries (3), variations in mortality rates between patient groups receiving similar treatments and residing in the same region of the world are observed (1, 4-6). Mortality is a measure influenced by many intrinsic host and pathogen factors (as well as host-pathogen interaction factors). Thus, we need better proxies to understand *C. neoformans* pathogenesis in patients and identify factors determining clinical outcome.

Although *C. neoformans* pathogenesis in the mouse model of cryptococcosis has been extensively studied, the correlation between disease characteristics in the human patient and clinical isolate infection in mice is unknown. The mouse model has been used to define *C. neoformans* virulence factors that allow the organism to be pathogenic, such as capsule, melanin, titan-cell formation, etc. The progression of disease in the inhalational mouse model - from initial inhalation into the lungs, to disseminated disease, and ultimately death due to central nervous system infection – is similar to human infection. Yet whether the mouse model accurately recapitulates differences in human disease, and whether the model can be used to identify subtle variations in virulence factors that impact the outcome of human disease remains

unexplored. Having clinical information from patients, we investigated the association between disease parameters in patients compared to mice infected with the same *C. neoformans* isolate.

Previous studies showed that pathogen-specific characteristics, such as genotype or the degree of antigen shedding, influence immune responses to *C. neoformans* and the clinical outcome of infected patients (5, 7-9). Multi-locus sequence typing (MLST) of 7 genetic loci has been used previously to identify genetically similar strains (5-10). For example, studies of clinical isolates in both Uganda and Brazil showed higher patient mortality associated with sequence type 93 (ST93) strains (5, 10). ST93 strains had increases in type 2 cytokines in *ex vivo* cytokine release assays, suggesting that these strains may shift the Th1/Th2 immune balance (5). Similarly, studies from Vietnam have identified an association between ST5 and infections in non-HIV patients but the underlying biological differences remain unknown (11). To explore the association between genotype and clinical outcome, we examined individual patient differences in outcome and immune response in patients infected with the same ST type. We found human patients infected with *C. neoformans* strains with the same sequence type can have dramatically different clinical outcomes. This dichotomy in disease outcome was recapitulated in the mouse model of cryptococcosis, suggesting these differences in clinical outcome were due to strain-specific characteristics.

Materials and Methods

Ethical Statement

Animal experiments were done in accordance with the Animal Welfare Act, United States federal law, and NIH guidelines. Mice were handled in accordance with guidelines defined by the University of Minnesota Animal Care and Use Committee (IACUC) under protocol 1308-30852A.

The study population consisted of human immunodeficiency virus (HIV)-infected, antiretroviral therapy (ART)-naive individuals with a first episode of cryptococcal meningitis screened for the Cryptococcal Optimal ART Timing (COAT) trial (clinicaltrials.gov: NCT01075152) (12). Participants were enrolled from two countries; Uganda (Mulago Hospital in Kampala and Mbarara Hospital in Mbarara) and South Africa (GF Jooste Hospital in Cape Town) between November 2010 and April 2012. Written informed consent was obtained from all subjects or their proxy, and all data were de-identified. Institutional Review Board approvals were obtained from each participating site. A total of 106 patients that had culture-positive CSF for *Cryptococcus* and from which isolates were sequenced for genotypic analyses (5, 8) were included in this study.

Strains and media

Cryptococcus clinical isolates were colony-purified from the cerebrospinal fluid (CSF) specimens from patients enrolled in the COAT trial and multi-locus sequence type (MSLT) was determined previously (5, 8). The strains used in this study are listed in Table 1. Strains were stored in glycerol stocks at -80°C. Strains from glycerol stocks were grown on YPD (yeast-

peptone-dextrose) agar for 24-48 h. *C. neoformans* cells from agar plates were transferred to YPD broth and grown overnight at 30°C with shaking before being used in experiments.

Mouse infection

C. neoformans strains were cultured overnight in YPD broth at 30°C. After incubation, *C. neoformans* cells were washed 3 times in sterile PBS, enumerated by hemocytometer and resuspended in sterile PBS at a concentration of 1 X 10^6 cells/ml. Groups of 6-8 week-old female A/J mice (Jackson Laboratory, Bar Harbor, Maine) were anesthetized by intraperitoneal pentobarbital injection. 10 mice per strain were infected intranasally with 5 X 10^4 cells in 50 µl PBS. Animals were monitored daily for morbidity and sacrificed when endpoint criteria were reached. Endpoint criteria were defined as loss of 20 % total body weight, loss of 2 grams in 2 consecutive days, or symptoms of neurological disease. Mice that survived to 150 days post-infection without exhibiting signs of disease were sacrificed and their tissues processed to determine lung, spleen and brain fungal burdens.

Tissue burden analysis

Terminal lung, spleen and brain tissues were collected at the time of mouse sacrifice to determine organ fungal burden. Collected tissues were homogenized in 2 ml PBS. Serial dilutions of tissue homogenates were plated on YPD medium supplemented with 0.04 mg/ml chloramphenicol and incubated at 30°C. *C. neoformans* colonies were counted after 48 h of incubation. Data presented are representative of 2-8 mice per strain.

Histopathology

Terminal lungs, spleen and brain were harvested from infected mice, fixed in 10% buffered formalin, paraffin-embedded, sectioned, and stained with H&E (hematoxylin and eosin). Tissue

sections were examined for cell size and morphology by microscopy. Data presented are representative of 1-4 mice per strain.

In vivo titan cell analysis

For a subset of infected mice, lungs were lavaged three times with 1.5 ml sterile PBS using a 20-gauge needle placed in the trachea. Cells in the lavage fluid were pelleted at 15,000 g and washed three times with PBS. Yeast cells were fixed with 3.7 % formaldehyde at room temperature for 40 min, washed 3 times with sterile PBS, and then resuspended in 200 µl sterile PBS. Cell body and capsule sizes were analyzed by microscopy. To visualize the yeast cells, a drop of india ink was added to the cell suspension on the slide and observed using a Zeiss Axioplan microscope (Axioimager, Carl Zeiss, Inc.). Cell body size was determined as the diameter of the yeast cell body and did not include the capsule. Data presented are representative of 1-3 mice per strain and a total of 200-400 *C. neoformans* cells per mouse were analyzed.

In vitro titan cell analysis

To induce the formation of titan cells *in vitro*, two previously described methods were used (13, 14). For the first method (13), *C. neoformans* cells from a YPD agar plate were transferred to 10 ml YPD broth medium in a T25cm3 flask (TPP, Switzerland), grown at 30°C for 22 h with shaking (150 rpm), washed with sterile double distilled water and cell number enumerated with a hemocytometer. Aliquots of 1 X 10^6 cells in 1 ml of minimal media (15 mM glucose, 10 mM $MgSO_4$, 29.4 mM KH_2PO_4, 13 mM glycine, 3 µM Vit B1, pH5.5) were transferred into 1.5 ml Eppendorf tubes and incubated in a thermomixer at 30°C with shaking (800 rpm) for 48 hours. For the second method (14), yeast cells were grown overnight at 30°C, 150 rpm in 2 ml YNB without amino acids and supplemented with 2% glucose. Yeast cells from the overnight culture

were washed twice with sterile PBS, cell number enumerated with a hemocytometer and resuspended in 0.5 ml PBS supplemented with fetal calf serum (FCS, ThermoFisher Scientific) to a final concentration of 1 X 10^3 cells/ml in each well of a 24 well plate. The plates were then incubated at 37°C 5% CO_2 for 48 h. After incubation, cell morphology was analyzed by microscopy (AxioImager, Carl Zeiss, Inc.). Cell body diameter was measured as described above. For *in vitro* titan cell analysis data presented are from 2 biological replicates per strain with 300 cells counted for each replicate.

Capsule formation

Capsule formation was analyzed from *C. neoformans* cells generated from (a) *in vitro* titan cell formation as described above, and (b) *C. neoformans* cells grown in DMEM media supplemented with fetal calf serum (FCS). For capsule induction in DMEM, *C. neoformans* cells were grown overnight in YPD at 30°C with shaking (250rpm), washed with sterile PBS and counted with a hemocytometer. Yeast cells were then inoculated at a final concentration of 1x 106 cells/ml into DMEM media supplemented with 10% FCS and incubated at 37°C, 5% CO_2 for 5 days. After incubation, yeast cells were fixed with 3.7% formaldehyde and analyzed by microscopy (Axioimager, Carl Zeiss, Inc.). Capsule width was defined as the difference between the diameter of the whole yeast cell (cell body and capsule) and the cell body diameter (no capsule) divided by two. Data presented are averages from a total of 100 *C. neoformans* cells per strain.

Drug resistance assays

Drug resistance assays were as described previously (15). Briefly, a microdilution assay was performed according to CLSI guidelines using 2.5×10^3 CFU/ml. Fluconazole and Amphotericin B were tested as 2-fold dilutions from 512 μg/ml to 0.0625 μg/ml and from 8 μg/ml to 0.0315

μg/ml, respectively, in a final volume of 200 μl per well. Spectrophotometric analysis of well turbidity at 600 nm was used to determine the minimum inhibitory concentration (MIC) for each strain. Plates were scanned in a Biotek Synergy H1 hybrid reader (Winooski, VT) prior to and after 72 h of incubation at 37°C. The amphotericin B MIC was defined as the drug concentration at which no growth was observed at 72 h (100% inhibition of growth). Fluconazole MIC was defined as a 50% reduction in growth (turbidity) compared to the no-drug control.

Statistical analysis

T-test was used to compare clinical parameters between patients who lived and those who died. A Fisher exact test was used to analyze the association between survival in mice and survival in human patients. *P*-values < 0.05 were considered significant.

Results

C. neoformans isolates with the same sequence type were associated with both mortality and patient survival

The association between clinical outcome and *C. neoformans* MLST-based sequence type (ST) is controversial, with some studies showing differences in patient clinical outcomes associated with specific sequence types and others showing no correlation (5, 7-9, 16). To explore this phenomenon, we examined individual patient mortality/survival in HIV+ cryptococcal meningitis patients (enrolled in the Cryptococcal Optimal ART Timing (COAT) clinical trial) that were infected with *C. neoformans* strains of the same sequence type (Fig 1A). In this cohort, 11 sequence types were observed in only one patient and were cumulatively analyzed as "unique ST" types. We observed a combination of patient death within 10-weeks (early mortality) and patient survival for eight of the sequence types (ST5, ST31, ST77, ST65, ST93, ST2, ST206 and ST187). No patients survived infections with ST40, whereas all patients survived the ST23 and ST71 infections (Table 2). While ST40 and ST23 patient mortality/survival were examples of the two ends of the spectrum, patient mortality (# patients with mortality due to cryptococcosis / total patients) differed across the sequence types (Figure 1A and Table 2). For example, ST187 had a high rate of patient mortality (i.e. low survival) whereas ST5 had a lower rate of patient mortality. Similar patient mortality/survival was observed with the unique sequence types.

These data show that the majority of sequence types had patients that succumbed to the *C. neoformans* infection and patients that survived the infection. There are a number of parameters, both patient and fungal, that could explain this difference in patient survival within

the same sequence type. We investigated factors that could impact whether a patient survived or succumbed to cryptococcal infection with the same sequence type.

Association between HIV disease and mortality/survival

C. neoformans infections are predominantly observed in patients with compromised immune function. To test the hypothesis that differences in the underlying HIV infection account for the observed differences in mortality/survival in the various sequence types, we examined CD4+ counts, cerebrospinal fluid white blood cell count (CSF_WBC), and HIV viral load in patients that died from cryptococcosis within 10-weeks vs. those that survived (Fig 2A-C). ST93 was the most prevalent sequence type, with 60 patients, and the only sequence type with enough patients to perform statistical analyses. Therefore, we also analyzed HIV viral load, CSF_WBC, and CD4+ cell count in the cohort of patients infected with ST93 strains alongside analyzes for all patients (Fig 2D-F). The number of CD4+ cells, CSF white blood cells and the HIV viral load was similar in patients that lived and those that died (p-value > 0.05). Similar results were observed when considering only the strains that were tested in the mouse model of cryptococcosis (Supplemental Figure 1).

Although the CSF_WBC counts did not differ between patients who died and those that survived the infection in the total cohort, patients who survived the ST93 infection had lower CSF_WBC counts compared to patients who died from the ST93 infections (Fig 2E, p-value < 0.05). These data suggest that observations at the population level do not always represent individual sequence types, and that higher WBC count at the time of presentation is associated with mortality in the ST93 infected patients.

Human mortality is associated with fungal burden in the CSF

To explore the hypothesis that fungal factors impact mortality/survival in the various sequence types, we analyzed the initial CSF fungal burden (CSF_CFUs), the amount of cryptococcal antigen in the CSF (CrAg) and the rate of fungal clearance in the patient CSF (EFA). Our analysis revealed that these fungal parameters partially correlate with patient survival (Fig 3). Looking at all patients individually, we see that patients with comparable cryptococcal CFUs have different clinical outcome (Fig 3A). However, lower CFUs were associated with survival in the ST93 infected patients (Fig 3D). We observed similar trends for the amount of cryptococcal capsular antigen detected in the patient CSF and the rate of fungal clearance (Fig 3B, E and C, F) with higher antigen levels and lower clearance associated with mortality in ST93 genotypes but not in the entire population. These data suggest that fungal parameters such as the ability to multiply inside the host, resistance to treatment and amount of antigen shedding by *Cryptococcus* cells play a role in infection outcome in a sequence type dependent manner.

When only the patients infected with strains that were tested in the mouse model of cryptococcosis were considered, only the fungal burden in the CSF was associated with virulence (Supplemental Fig 2 A, D). The cryptococcal antigen detected in the patient CSF as well as the rate of fungal clearance were similar between the patients infected with high, intermediate and low virulence strains (Supplemental Fig 2 B, E and C, F).

C. neoformans virulence in human patients correlates with virulence in a mouse model of cryptococcosis

After observing that both human and fungal parameters were partially associated with patient survival, we determined whether differences in *Cryptococcus* virulence in humans could be recapitulated in the mouse inhalation model of cryptococcosis. We selected paired strains – one from a patient that died and one from a patient that lived – from ST5, ST31, and ST77 for analysis in the mouse model (Fig 1B). In addition, we analyzed two high-mortality ST40 strains, two high-mortality ST93 strains, and one late-mortality ST93 strain (Fig 1B). Mice infected with the clinical strains showed variation in disease progression in the mouse inhalation model (Fig 4). Based on mouse survival, the clinical strains were divided into three groups: high, intermediate and low virulence strains (Figures 4A-C). The high virulence strains were defined as strains that caused 80% mortality within 37 days post-infection (Fig 4A). The intermediate virulence strains had mortality between 69-129 days post-infection (Fig 4B). The low virulence group consisted of three strains; mice infected with two strains, UgCl552 and SACl010, showed no overt signs of the disease at 150 days post-infection, and the third strain, UgCl223 had reduced virulence where 60% of mice survived through 150 days post-infection (Fig 4C). By histologic examination of lungs, no cryptococci were identified in UgCl552 or SACl010, however UgCl223 had a low to moderate number of organisms within the lung and one of two mice examined had infiltration of small cryptococcal organisms into the brain with necrosis in affected areas.

We next compared the death/survival of patients and mice infected with the same *Cryptococcus* strain (Figure 5 and Table 3). All but two of the isolates associated with early mortality in humans showed high virulence in mice (Figure 5A). Strains SACl010 and UgCl552

had low virulence in mice but had an early mortality in humans. Histological examination of lungs from 2 mice infected with SAC1010 revealed low numbers of cryptococci within alveoli that were eliciting a strong pulmonary inflammatory response. Interestingly, while the human patient infected with SACl010 died 41 days after diagnosis, the clinical records indicated that the patient died from Immune Reconstitution Inflammatory Syndrome (IRIS) and not cryptococcosis. IRIS is defined as detrimental inflammatory responses despite fungal clearance (e.g. negative cultures) (17), consistent with the inflammation observed in the mouse model. The patient infected with strain UgCl552 cleared the initial infection but then died at 99 days post-diagnosis of an unknown cause at home; again, consistent with the mouse model showing low virulence of the *Cryptococcus* strain.

All strains associated with late mortality or survival in human patients showed reduced virulence in mice (Figure 5 and Table 3). No strain had high virulence in mice but low virulence in humans, providing further support for the association between human and mouse virulence. When all 10 strains (including the outliers SACl010 and UgCl552) were considered, a trend between human and mouse survival was observed (Figure 5A, R^2= 0.2201). However, when both outliers are removed, the association between human and mouse survival is robust (Figure 5B, R^2= 0.7895). These data show human and mouse mortality are similar when infected with the same *C. neoformans* strain, although unrelated human causes of mortality can influence the human clinical outcome.

Mouse virulence is associated with fungal burden

To further investigate the factors that influence infection outcome in mice, we compared mouse survival with tissue fungal burden at sacrifice. High virulence strains were associated

with high fungal burdens while intermediate and low virulence strains correlated with low CFUs in lungs of infected mice (Fig 6A). Similar trends were observed for fungal burdens in the spleen, but differences were not statistically significant (Fig 6B). We observed similar terminal fungal burdens in the brains of mice infected with high and intermediate virulence strains, and lower fungal burdens in the mice infected with low virulence strains sacrificed at 150 days post-infection (Fig 6C). These data suggest that both high and intermediate virulence strains can disseminate to the brain, the only difference being the time it takes to reach the brain tissue (Fig 6C and Table 3). Histologic examination of lung and brain from two intermediate strains UgC1425 and SAC1059 revealed moderate to large numbers of cryptococci and cryptococcoma formation with a moderate to strong inflammatory response.

Mice infected with low virulence strains showing no overt disease were sacrificed at 150 days post-infection. These mice had moderate CFUs in the lungs but low CFUs in the spleen and brain, suggesting that the low virulence of these strains was due to reduced fungal dissemination (Fig 6 B-C). In addition, we observed differences in the disease progression/pathogenesis in mice infected with the three low virulence strains. One of the low virulence strains (UgCl223) had high terminal lung CFUs ($1X\ 10^7$) and very few CFUs in the spleen (10) and brain (10). Histologic examination of lung and brain from 2 additional animals revealed low numbers of cryptococci in the lung with moderate inflammation, and one mouse had small cryptococci infiltrating the meninges, scattered in small areas of necrosis within the neuropil. These data suggest that UgCl223 can multiply in the lungs and may disseminate to the brain but may not remain viable in the brain. Most mice infected with the other two low virulence strains (SACl010 and UgCl552) cleared the infection. Seven out of nine mice infected with SACl010 had no CFUs in the lung, spleen or brain tissues at 150 days post-infection. Five out of nine mice

316 infected with UgCl552 had no CFUs in the lungs and only one mouse with lung CFUs had
317 detectable CFUs in the spleen or brain.

318 **_C. neoformans_ virulence did not correlate with known virulence factors in _in vitro_**
319 **conditions**

320 After observing that infection outcome was strain-specific in both human and mouse
321 infection, we tested whether production of virulence factors previously shown to be associated
322 with *C. neoformans* pathogenesis (18-23) were associated with survival in our study. Virulence
323 factors tested include the ability to growth at high temperature (37°C), capsule formation, and the
324 ability to form titan cells both *in vitro* and *in vivo*. Virulence did not correlate with the ability to
325 grow *in vitro* in either nutrient-rich and limited nutrient conditions, at low and high temperatures
326 (30°C and 37°C) (Supplemental Figure 3). Using two previously described methods (13, 14), we
327 found that virulence was also not associated with the ability to form titan cells *in vitro*
328 (Supplemental Table 1). However, we observed a trend where low virulence strains formed more
329 titan cells than high and intermediate virulence strains when grown in DMEM media
330 supplemented with serum (Table 4). Finally, we also analyzed *in vitro* capsule formation. While
331 capsule varied across the strains, there was no difference in the size of the capsule between low
332 and high virulence strains (Supplemental Figure 4).

333 We next compared the susceptibility of the clinical strains to the antifungal drugs
334 fluconazole and amphotericin B, which were used to treat the CM-patients in our cohort. The *in*
335 *vitro* susceptibility to the antifungal drugs fluconazole and amphotericin B was not associated
336 with strain virulence (Supplemental Table 2). Next, we analyzed the levels of heteroresistance to
337 fluconazole, a factor that was previously associated to *C. neoformans* virulence (24), as well as

the ability to grow in the presence of cell wall stressors calcofluor white (CFW), congo red and caffeine. We observed a trend where high virulence strains manifested heteroresistance at high levels of fluconazole and low virulence strains developed heteroresistance at low levels of fluconazole (Table 4). However, not all high virulence strains had high levels of fluconazole heteroresistance. Two highly virulent strains, SACl012 and KN99α, manifested fluconazole heteroresistance at 8 and 16 μg/ml respectively, while the other three high virulence strains showed heteroresistance at 32 μg/ml (Table 4). In addition, high virulence strains grew better in the presence of cell wall stressors compared to two of the three low virulence, SACl010 and UgCl552 (Table 4). However, the low virulence strain UgCl223 did not show any growth defect in any of the tested conditions (Table 4). Our *in vitro* assays did not identify a single condition or a virulence-determining factor that exclusively distinguishes our high and low virulence strains, but there was a trend where most of low virulence strains grew slower or did not grow in the presence of various stresses.

Discussion

In this study, we showed that patients infected with *C. neoformans* strains of the same sequence type could have different clinical outcomes. Using a mouse inhalation model of cryptococcosis, we showed that these differences in virulence were strain-specific – with similar mortality rates observed in both humans and mice infected with the same strain. In both humans and mice, we identified an association between increased fungal burden and early mortality, but no single *in vitro* virulence factor could explain the observed *in vivo* differences between closely related strains.

We sought to identify factors associated with infection outcome, both in humans and infected mice, which could explain the observed differences in mortality between *C. neoformans* strains with the same sequence type. We found that *C. neoformans* growth *in vivo* was associated with mortality rate in mice and humans. Our observations are in accord with previous clinical trials showing that CSF fungal burden and the rate of fungal clearance were associated with patient mortality (25-28). However, underlying human immune deficiencies did not correlate with patient outcome. Some patients died, others survived the infection, while having similar CD4+ cell counts, HIV viral load and white blood cell numbers in their CSF. These data are also consistent with previous observations that baseline CD4+ cells, CSF white blood cell counts, and HIV viral load do not influence the mortality of HIV-CM patients (29-31). Taken together, these data show that differences in patient outcome are due, in part, to differences in the strains of *C. neoformans* with which the patients are infected. A previous study from our group showed that differences in *C. neoformans* genotype influence patient immune response and clinical outcome in HIV-CM patients (5). To further explore this phenomenon, we examined the impact of sequence type on patient outcome. We found that patients infected with *C. neoformans* isolates

374 of the same sequence type had differential clinical outcome, some patients survived while others
375 died from the infection.

376 To determine whether these differences in clinical outcome where inherent to the
377 individual *C. neoformans* strains, we tested the virulence of a subset of the clinical isolates in the
378 mouse inhalational model of cryptococcosis. We showed a robust association between patient
379 and mouse mortality for the majority of *C. neoformans* strains tested. These data not only show
380 that the mouse model accurately recapitulates human disease, but also strongly suggest that a
381 large proportion of patient mortality is due to inherent differences between the infecting *C.*
382 *neoformans* strains.

383 Several factors such as cellular phenotypes and stress response mechanisms have been
384 associated with *C. neoformans* virulence and pathogenesis in the mouse model of cryptococcosis
385 (18, 21, 22). In addition, a few studies showed that pleomorphism and the production of some
386 virulence factors under *in vitro* conditions correlated with infection outcome in human patients
387 (7, 23, 32). Therefore, we tested whether the ability to produce known virulence factors, as well
388 as other phenotypes previously associated with pathogenesis, correlate with the virulence level of
389 the subset of *C. neoformans* clinical isolates used in our study. The dominant virulence factors in
390 *C. neoformans* are high temperature growth and capsule formation (33, 34). High and low
391 virulence strains grew similarly at high temperature, both in nutrient-rich and low nutrient
392 conditions. We observed similar patterns in capsule formation where high and low virulence
393 strains produced capsule of similar size. However, the size of the capsule formed during
394 infection was significantly greater that the capsule induced *in vitro* suggesting differences in
395 capsule structure, composition and/or function (35-37). This might explain our observations and
396 those from other studies (38-40) that capsule size *in vitro* does not correlate with *C. neoformans*

virulence. Next, we tested whether increased virulence was associated with increased resistance to two antifungal drugs, fluconazole and amphotericin B, used to treat patients in our cohort. *In vitro* susceptibility to fluconazole and amphotericin B also did not correlate with virulence of our clinical strains. The lack of correlation between infection outcome and the susceptibility to antifungal drugs in *in vitro* assays can be explained by physiological differences between *in vitro* conditions and *in vivo* environments in which *C. neoformans* cells have to survive (41). We also tested the ability to form titan cells, a phenotype that has been associated with *C. neoformans* virulence/pathogenesis (18, 19, 42). Using recently described techniques to induce titan cells *in vitro* (13, 14), we found that the ability to form titan cells *in vitro* did not correlate with the degree of virulence of our clinical strains. Similar results were observed with *in vivo* titan cell formation in the mouse model.

Under *in vivo* conditions, *C. neoformans* cells have to withstand multiple host defense mechanisms. Our data show that, individually, the *in vitro* analyses we tested were unable to distinguish high versus low virulence strains. Thus, no single *in vitro* assay/condition can be used as a proxy for *in vivo C. neoformans* virulence. However, we did observe a trend when comparing across the various *in vitro* assays (Table 4). Increased virulence was associated with heteroresistance at high levels of fluconazole, ability to grow in the presence of cell wall stressors, and inability to form titan cells in DMEM and serum media. In contrast, strains with low virulence were unable to grow in the presence of cell wall stressors, had low fluconazole heteroresistance and formed more titan cells in DMEM and serum. Most mice infected with strains that were unable to grow in the presence of multiple stresses *in vitro* cleared the infection and the strains did not disseminate to the brain, showing that growth defects *in vitro* in response to multiple stresses might predict inability to multiply *in vivo* during infection. These

observations suggest that a panel of *in vitro* stresses could be developed to differentiate between high and low virulence *C. neoformans* strains. Future studies that incorporate a large number of clinical isolates are necessary to determine whether an appropriate panel of *in vitro* stresses can be identified to accurately predict *in vivo* virulence. If confirmed, this could be a valuable tool in clinical laboratories to help in the early identification and follow up of patients infected with high virulence strains and at high mortality risk.

An alternative explanation for differences in *in vivo* virulence that do not correlate with single *in vitro* assays is that the observed differences are due to novel virulence factors. *In vivo* studies with evolutionarily closely related strains, such as those presented here with identical ST types, need to be combined with genomic analyses to identify novel genes that are critical *in vivo*. Our observation that strains with identical sequence type can have dramatically different virulence in both humans and the mouse model suggest that multi-locus sequence type does not have sufficient resolution to identify the underlying differences between strains – whole genome sequencing of closely related strains, and at the population level, may be required to identify these *in vivo* virulence factors.

In summary, our study had two major findings. First, the mouse inhalation model of cryptococcosis accurately recapitulates human infection. Thus, this model can be used to explore how differences between *C. neoformans* clinical isolates impact human disease outcome. Second, *C. neoformans* isolates from the same sequence type were associated with different clinical outcomes. The association between sequence type and clinical outcome has been contentious, with some patient cohorts showing no association and some with robust associations (5, 7, 43, 44). Our data show that belonging to the same lineage or sequence type does not necessary mean *C. neoformans* strains will have a comparable degree of virulence. The

443 virulence-determining factor(s) is not the lineage/sequence type of the *C. neoformans* strain, but
444 instead other genotypic or phenotypic characteristics specific to individual isolates within the
445 sequence type. Future studies investigating the relationship between individual *C. neoformans*
446 genotypes and virulence are needed to fully understand pathogen-associated factors that
447 influence *in vivo* virulence and ultimately clinical outcome.

448

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Table 1. Strains used in this study

Strain	Sequence type	Source/Reference
KN99α	ST2	United States of America [45]
UgCl223	ST31	Kampala, Uganda [8]
UgCl302	ST93	Kampala, Uganda [8]
UgCl387	ST31	Kampala, Uganda [8]
UgCl395	ST93	Kampala, Uganda [8]
UgCl425	ST77	Kampala, Uganda [8]
UgCl538	ST93	Kampala, Uganda [8]
UgCl552	ST5	Kampala, Uganda [8]
MbCl006	ST77	Mbarara, Uganda [8]
SACl010	ST40	Cape Town, South Africa [8]
SACl012	ST40	Cape Town, South Africa [8]
SACl059	ST5	Cape Town, South Africa [8]

Table 2. HIV-CM patient infection outcome

	Sequence types												
	ST23	ST71	ST5	ST31	ST77	ST65	ST93	Unique ST	ST206	ST2	ST187	ST40	All patients
Early mortality (within 10 weeks)	0	0	2	1	2	1	19	4	1	2	2	2	36
Late mortality (after 10 weeks)	0	0	1	0	0	0	1	1	0	0	0	2	5
Survival	3	2	10	3	5	2	30	6	1	2	1	0	65
Total	3	2	13	4	7	3	50	11	2	4	3	4	106
Survival %	100.0	100.0	76.9	75.0	71.4	66.7	60.0	54.5	50.0	50.0	33.3	0.0	61.3

Table 3. Association between clinical strain virulence in human and mice

			Human infections		Mice infection	
	Strain name	Sequence type	Mortality (days from diagnosis)	Outcome	Mortality (Days to 80% mortality)	Outcome
High virulence	SACl012	ST40	NA	NA	18	All died
	KN99α	ST2	NA - WT	NA - WT	21	All died
	UgCl395	ST93	80	Died	30	Died (9/10)[1]
	UgCl302	ST93	32	Died	32	Died (9/10)
	UgCl387	ST31	36	Died	37	Died (9/10)[2]
Intermediate virulence	UgCl538	ST93	33	Died	65	All died
	SACl059	ST5	-	Survived	91	Died (9/10)[3]
	MbCl006	ST77	221	Died	92	Died (9/10)[4]
	UgCl425	ST77	-	Survived	129	Died (8/10)[5]
Low virulence	UgCl223	ST31	-	Survived	End of experiment	Survived
	UgCl552	ST5	99	IRIS	End of experiment	Survived
	SACl010	ST40	41		End of experiment	Survived

[1] Nine out of 10 mice died, with remaining mouse sacrificed after 150 days, terminal CFUs of surviving mouse were 6.4 X 10^5 cells in the lungs and $4x10^2$ in the brain tissues

[2] Nine out of 10 mice died, with remaining mouse sacrificed after 150 days, terminal CFUs of surviving mouse were 7 x 10^5 cells in the lungs, no *Cryptococcus* cells detected in the brain

[3] Nine out of 10 mice died, with remaining mouse sacrificed after 150 days, terminal CFUs were 6 x 105 cells in the lungs, no *Cryptococcus* detected in the brain

[4] Nine out 10 mice died, with remaining mouse sacrificed after 150 days, terminal CFUs were 6 x 10^6 cells in the lungs, and 6 x 10^3 cells in the brain

[5] Eight out of 10 mice died, with remaining mice sacrificed after 150 days, terminal CFUs were 4 x 10^5 cells in the lungs of each mouse and 4 x 10^4 cells in the brain of one mouse and 3.6 x 10^4 cells in the brain of the second mouse

NA: patient information not available, **NA-WT**: patient information not available, wild type laboratory control strain, **NI**: not identified at the time of death, patient died at home.

Table 4. Cellular phenotypes and growth in the presence of cell wall stressors

		Titan cells in DMEM+FCS (%)	Capsule size in DMEM+FCS (μm)	Fluconazole heteroresistance (μg/ml)	Growth on Congo Red	Growth on CFW	Growth on Caffeine
High resistance	KN99α	0	3.8	16	++++	++++	+++
	SACl012	0	2.5	8	+++	++++	++++
	UgCl302	6	5.0	32	++++	++++	++++
	UgCl395	0	7.5	32	+++	++++	++++
	UgCl387	0	7.4	32	+++	++++	+++
Intermediate resistance	UgCl425	0	3.4	32	++++	+++	+++
	UgCl538	1	5.8	16	+++	++++	++++
	SACL059	0	3.3	16	++++	+++	++++
	MBCL006	3	4.8	16	++++	++++	++++
Low resistance	UgCl223	1	3.1	16	++++	+++	++++
	UgCl552	4	3.1	4	-	-	-
	SACL010	10	6.3	16	-	-	++

“-” No growth observed, “+” number of dilutions that grew on agar media by spotting assay

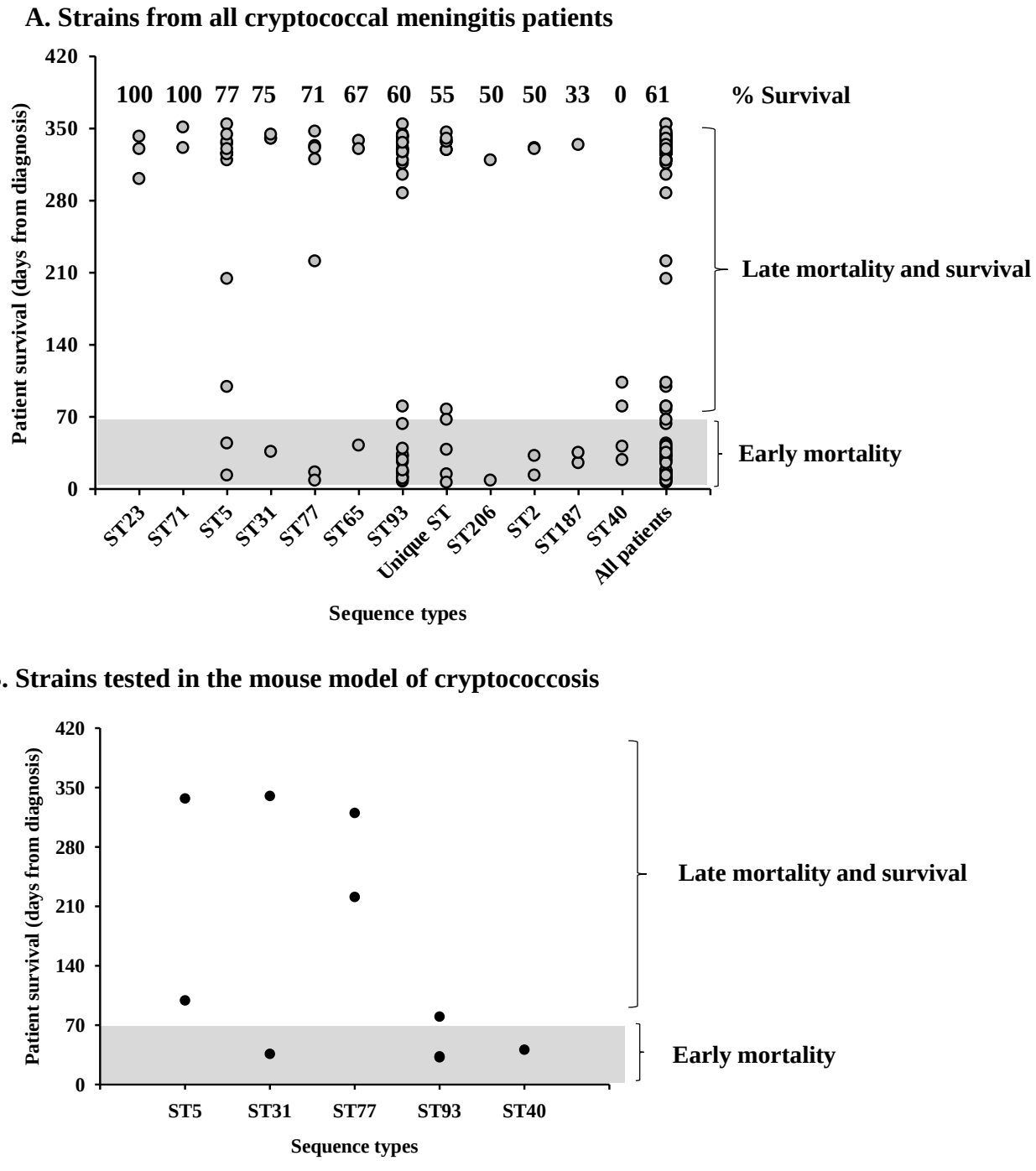


Figure 1. *C. neoformans* sequence types and human mortality. *C. neoformans* clinical strains were isolated from the CSF of HIV-CM patients, grown on YPD agar media at 30°C and stored at -80°C before their sequence types were identified. Early mortality: died within 10 weeks from diagnosis, late mortality: died after 10 weeks, survival: survived the infection. Unique ST; sequence types with only one patient.

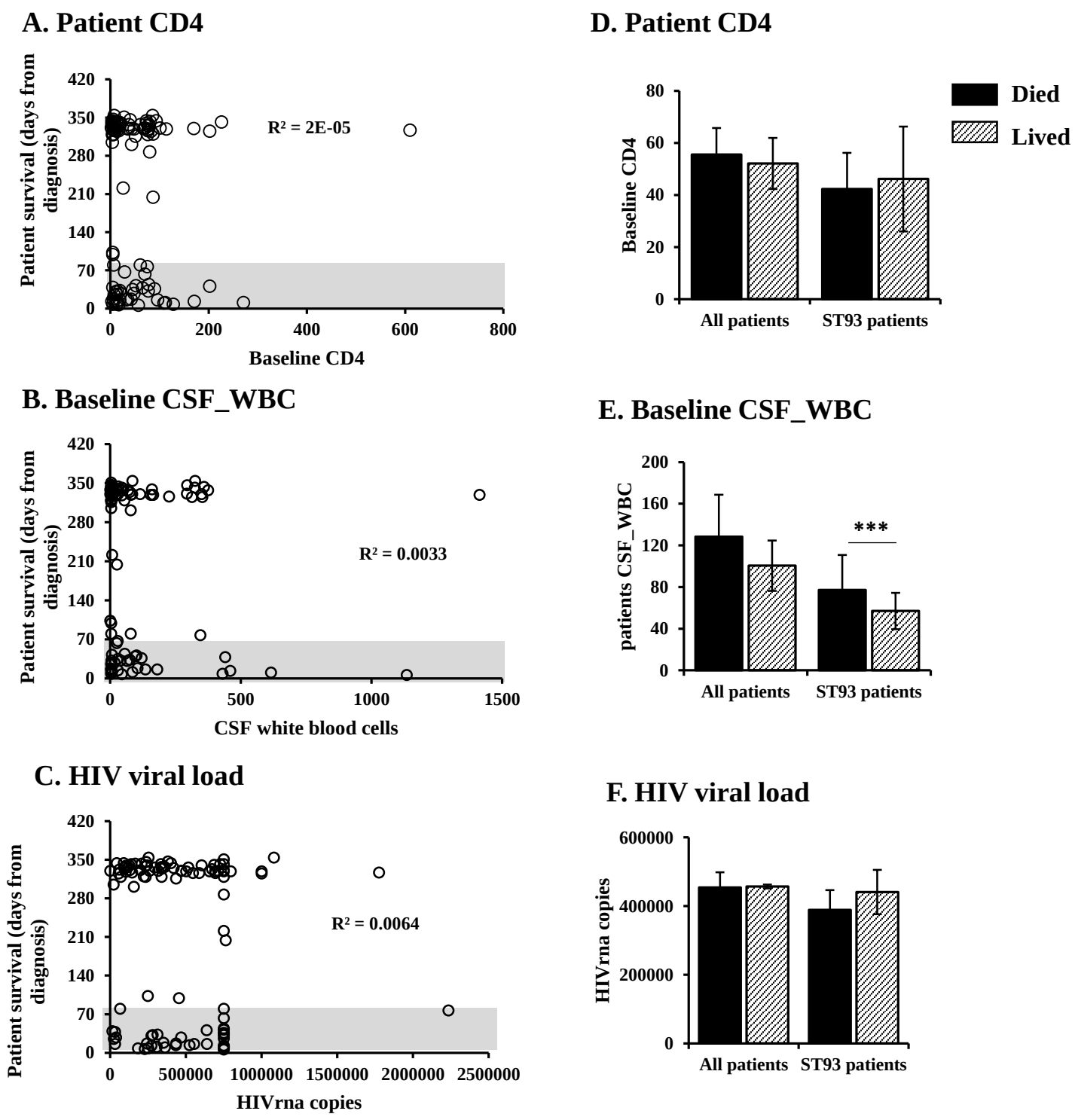


Figure 2. Human mortality is not determined by patient parameters. Clinical outcome was compared to baseline CM patient characteristics including CD4+ cells (A, D), white blood cell counts in the CSF (B, E), and HIV viral load (C, F). Graphs A-C show individual patient parameters plotted against survival from diagnosis. Gray bar indicates mortality within 10-weeks. Survival beyond 280 days is indicated as the discharge date from the clinical trial. Graphs D-F show patient parameters when classified as died (mortality) by 10 weeks post-diagnosis or lived past 10 weeks post-diagnosis. Error bars represent standard error of the mean. CD4 cells: CD4+ T helper cells, CM: cryptococcal meningitis, CSF: cerebrospinal fluid, HIV: human immunodefiency virus, RNA: ribonucleic acid, ST93: sequence type 93, WBC: white blood

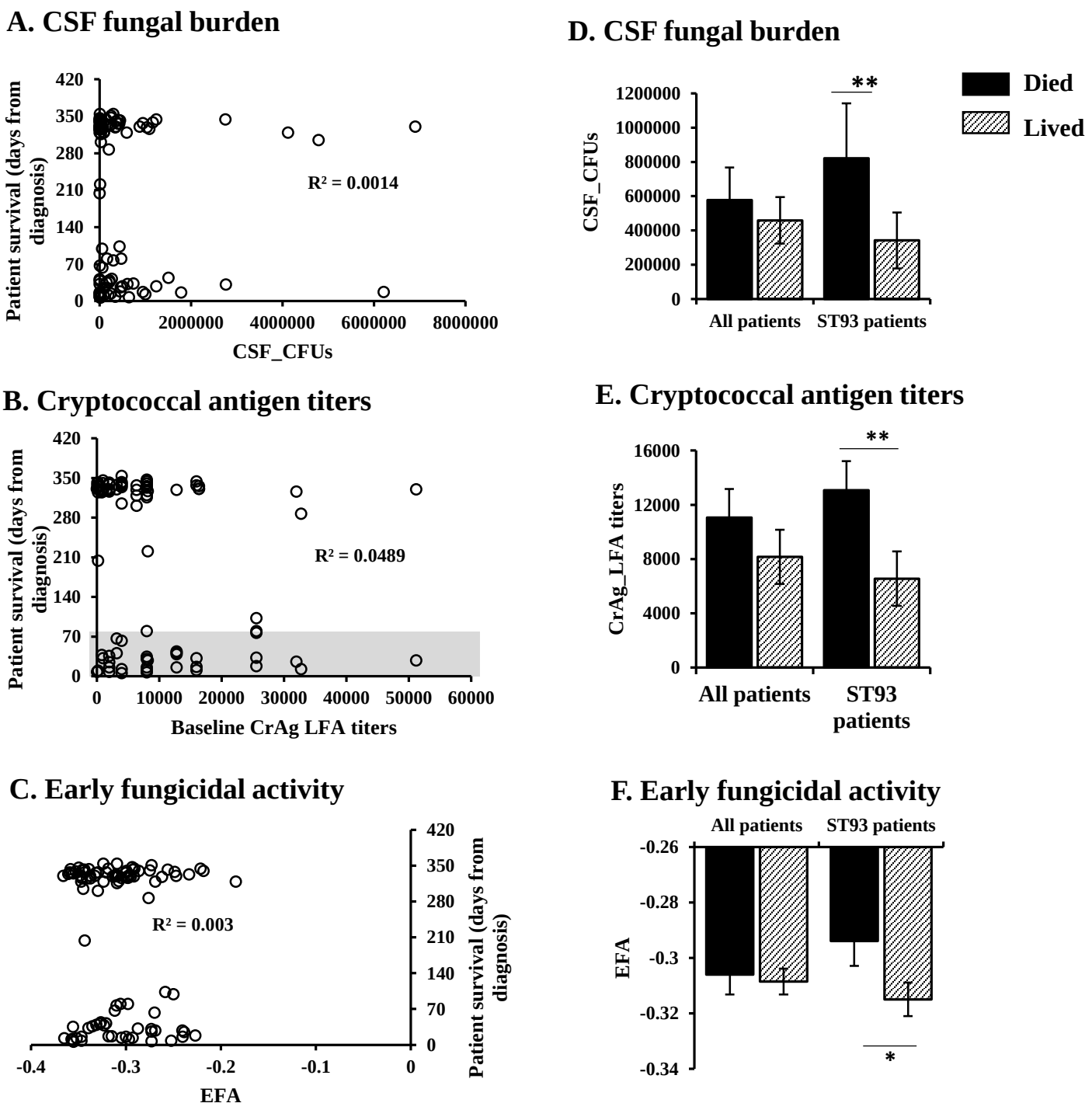


Figure 3. Patient survival is partly associated with fungal parameters. Clinical outcome was compared to fungal parameters including CSF-fungal burden determined by colony forming units (CFUs) (A, D), cryptococcal antigen titers in the CSF (B, E) and rate of fungal clearance determined by the early fungicidal activity (EFA) (C, F). Graphs A-C show individual patient parameters plotted against survival from diagnosis. Graphs D-F show patient parameters when classified as died (mortality) by 10-weeks post-diagnosis or lived (survived) past 10-weeks post-diagnosis. Error bars represent standard error of the mean. CFU: colony forming unit, CSF: cerebrospinal fluid, CrAg LFA: cryptococcal antigen lateral flow assay, EFA: early fungicidal activity, ST93: sequence type 93. *p< 0.05, **p< 0.01.

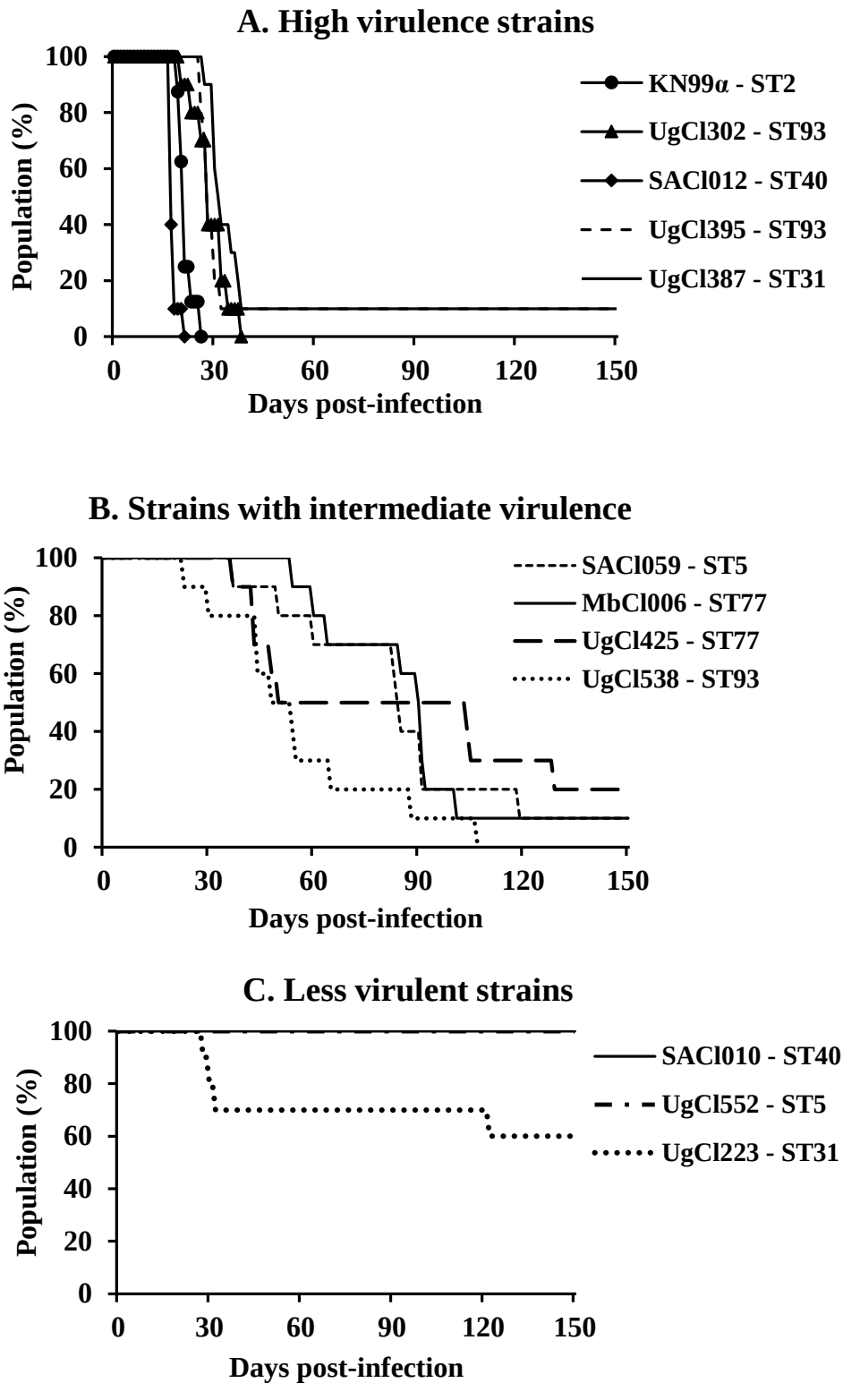


Figure 4. *C. neoformans* clinical strains show differential virulence in mice. Groups of ten 6-8 week old A/J mice were infected intranasally with 5 X 10^4 cells from *C. neoformans* clinical strains isolated from the CSF of HIV-CM patients. Progression to severe morbidity was monitored for 150 days and mice were sacrificed when end point criteria were reached. A) high virulence strains, B) strains with intermediate virulence, C) low virulence strains.

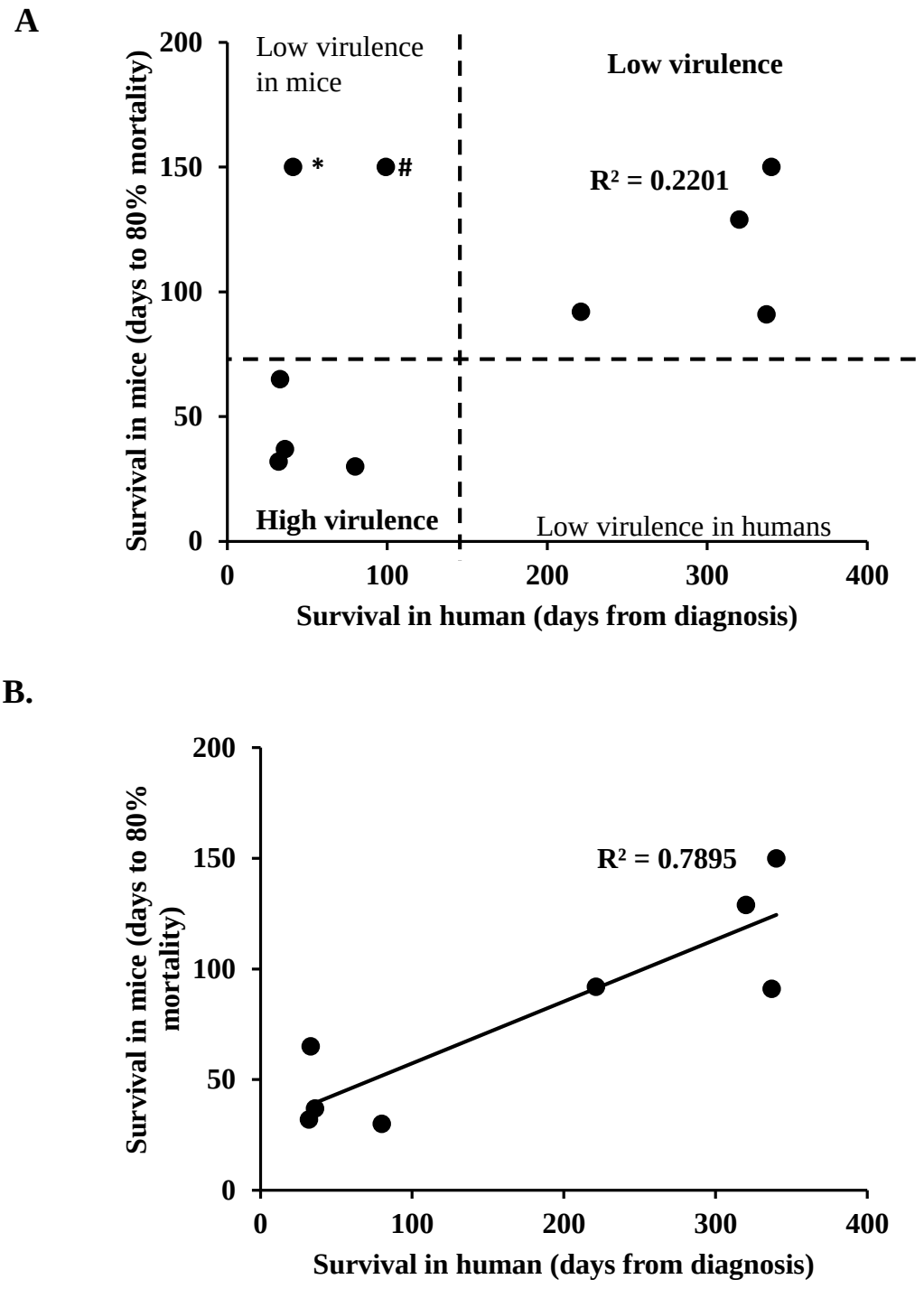


Figure 5. Infection outcome in human patients is associated with disease outcome in mice. Clinical outcome in human patients was compared to infection outcome in mice infected with the same *C. neoformans* strain. A) Correlation between human and mice survival for all 10 clinical strains. B) Correlation between human and mice survival when outliers (SACl010 and UgCl552 strains) were removed. * The SACl 010 strain was associated with low virulence in mice but the human patient died of IRIS at 41 days post-diagnosis. # The patient infected with UgCl552 died at home after clearing the initial *C. neoformans* infection. The cause of death was not determined.

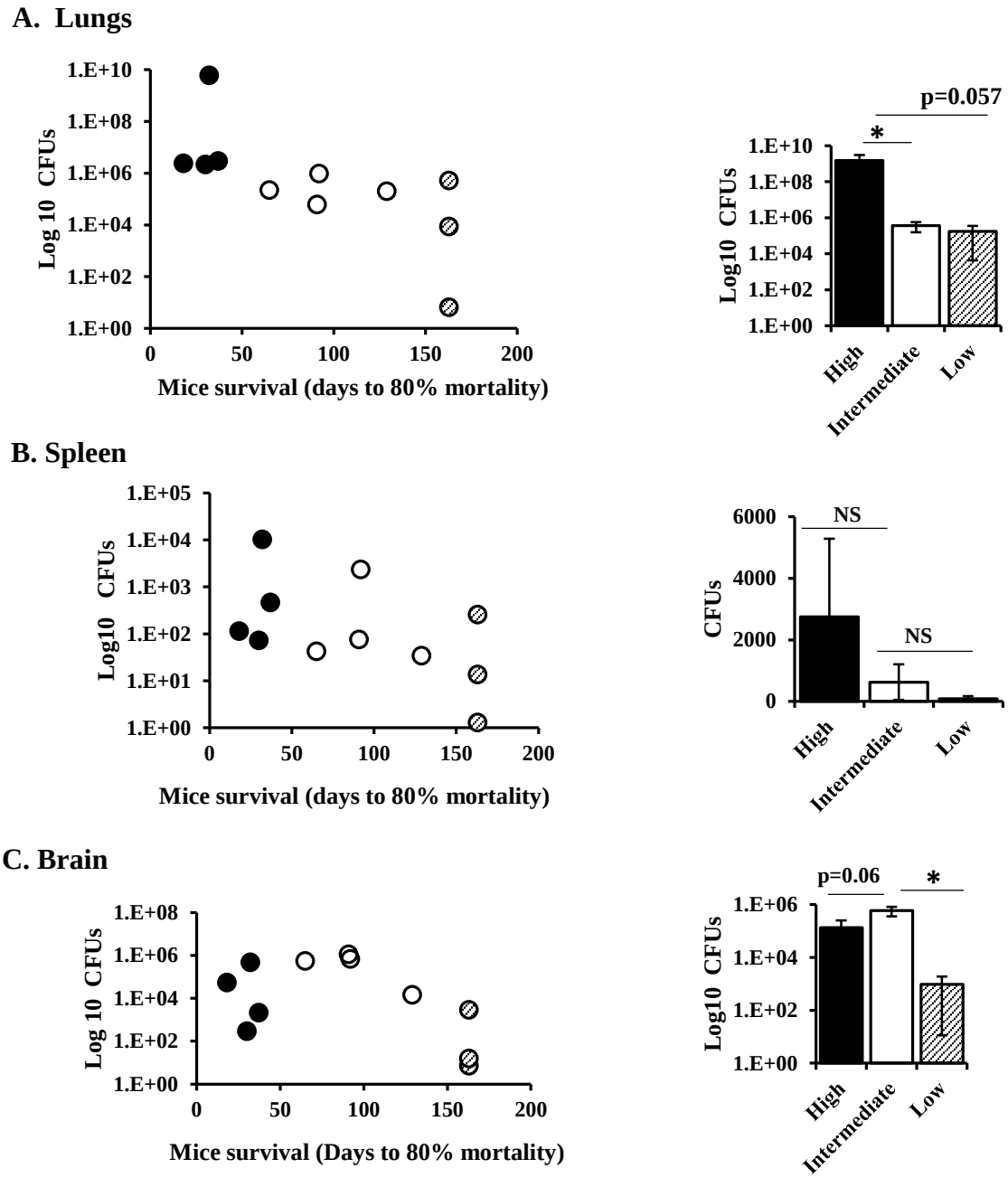


Figure 6. Mouse survival correlates with organ fungal burden. Groups of ten 6-8 week old A/J mice were infected intranasally with eleven *C. neoformans* clinical strains and a wild type laboratory strain. Progression to severe morbidity was monitored for 160 days, and mice were sacrificed at 160 days or when end point criteria were reached. Lungs (A), spleen (B) and brain (C) were harvested, homogenized and serial dilutions were plated on agar plates to determine tissue fungal burdens. Strains were classified in three groups as high, intermediate and low virulence strains as determined in the mice survival experiment. Left graphs show individual *C. neoformans* strains plotted as tissue fungal burdens against time to 80 % mortality of infected mice. Graphs on the right show average CFUs of high, intermediate and low virulence group strains. Error bars represent standard error of the mean. CFU: colony forming unit, NS: not statistically significant, PBS: phosphate buffered saline. * $p < 0.05$.

Supplemental Table 1. Titan cell formation

		In vitro		*In vivo*
		Protocol-1	Protocol-2	
High virulence	KN99α	+	+	+
	UgCl302	-	-	+
	UgCl387	-	-	+
	UgCl395	-	-	+
	SACl012	-	-	-
Intermediate virulence	UgCl425	-	-	+
	UgCl538	-	-	+
	SACl059	-	-	+
	MbCl006	-	-	+
Low virulence	UgCl223	-	-	+
	UgCl552	+	-	+
	SACl010	+	-	+

+: presence of titan cells, -: no titan cell observed

Supplemental Table 2. Minimum Inhibitory Concentration (MIC) for fluconazole and amphotericin B is not associated with strain virulence

	Strain	Fluconazole MIC	Amphotericin B MIC
High virulence	KN99α	1	0.5
	SACl012	1	0.25
	UgCl302	1	1
	UgCl395	2	0.5
	UgCl387	0.5	0.5
Intermediate virulence	UgCl425	2	1
	UgCl538	2	1
	SACl059	0.125	>8
	MbCl006	1	1
Low virulence	UgCl223	2	1
	UgCl552	0.5	0.5
	SACl010	2	ND

ND: Not determined, isolate did not grow

Supplemental Table 3. Mice lung histology

	Strain	Airways	Bronchial Epithelium/ Lamina Propria	Central Alveoli	Inter-alveolar septa	Peripheral Alveoli	Granuloma
High virulence	Non-infected	-	-	-	-	-	-
	KN99α	+	-	+++	+	+++	+++
	SACl012-1	+/++	-	++	-	++	+/++
	SACl012-2	+/++	-	++	-	+	++
	SACl012-3	++	-	++	-	+/++	++
	SACl012-4	++	-	+++	-	+	++
	UgCl302-1	+	+	+++	-	++/+++	++
	UgCl302-2	+	-	+++	-	+++	+++
	UgCl387-1	+	+	+++	-	++	++
	UgCl387-2	++	-	+++	-	++	++
	UgCl395-1	+	-	+++	-	++/+++	++
Intermediate virulence	UgCl538-1	++	-	+++	-	+++	-
	UgCl538-2	-	-	++	-	+	-/+
	UgCl425-1	++	+++	++	++	++	++
	UgCl425-2	+	++	++	++	++	+++
	SACl059-1	+/++	++	++	++	++	+++
	MbCl006-1-1	-	-	+/++	-	+	++
	MbCl006-1-1	+	-	+/++	-	+	++
Low virulence	UgCl552-1	-	-	-	-	-	-
	UgCl552-2	-	-	-	-	-	-
	UgCl223-1	+	++	+	++	+	+/++
	UgCl223-2	-	-	+	-	+	+
	SACl010-1	-	-	+	-	-/+	-/+
	SACl010-2	-	-	+	-	+	-/+

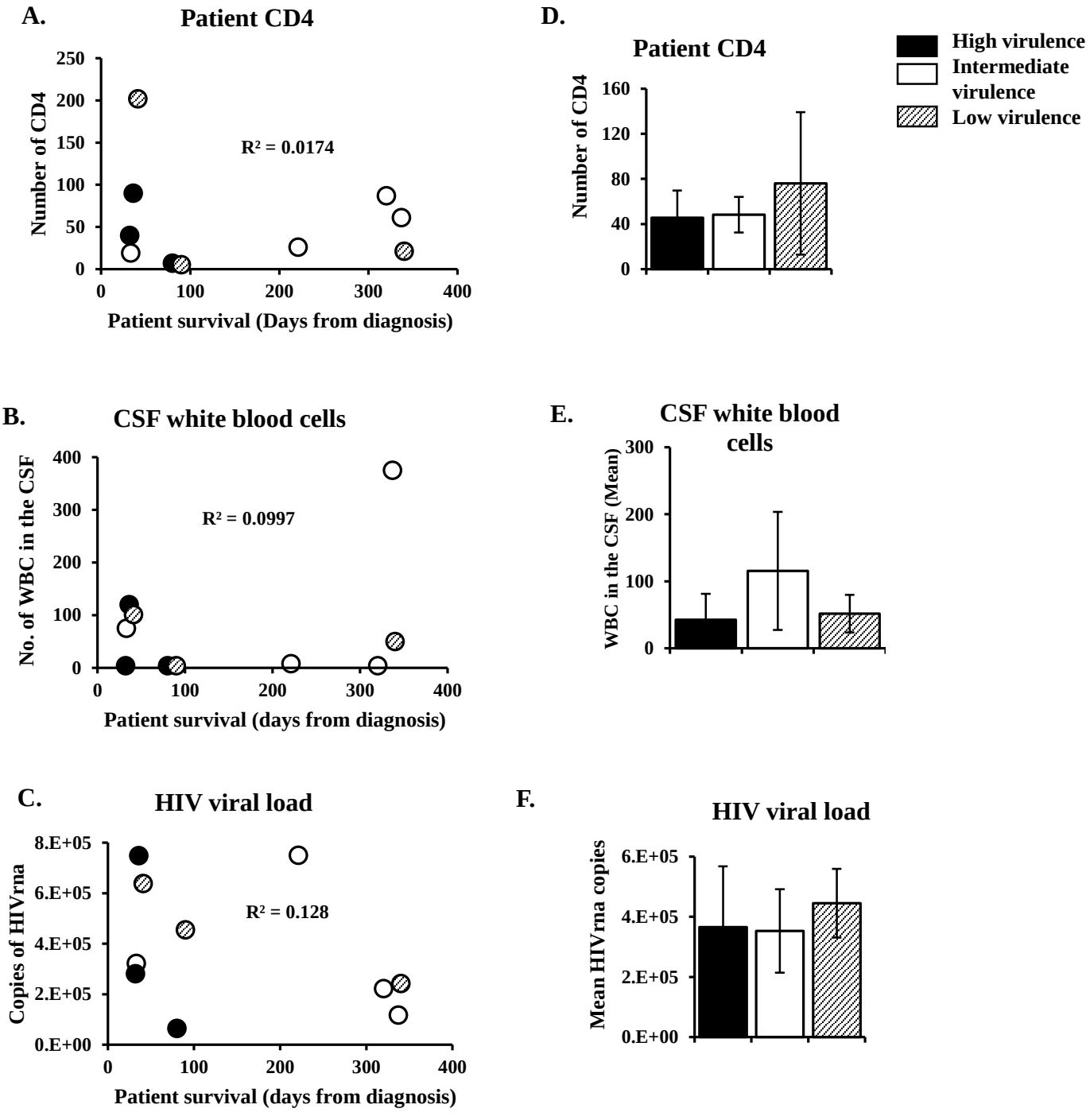
Presence of *Cryptococcus*: - none, + scattered, ++ multi-focal, +++ confluent

Supplemental Table 4. Mice Brain Histology

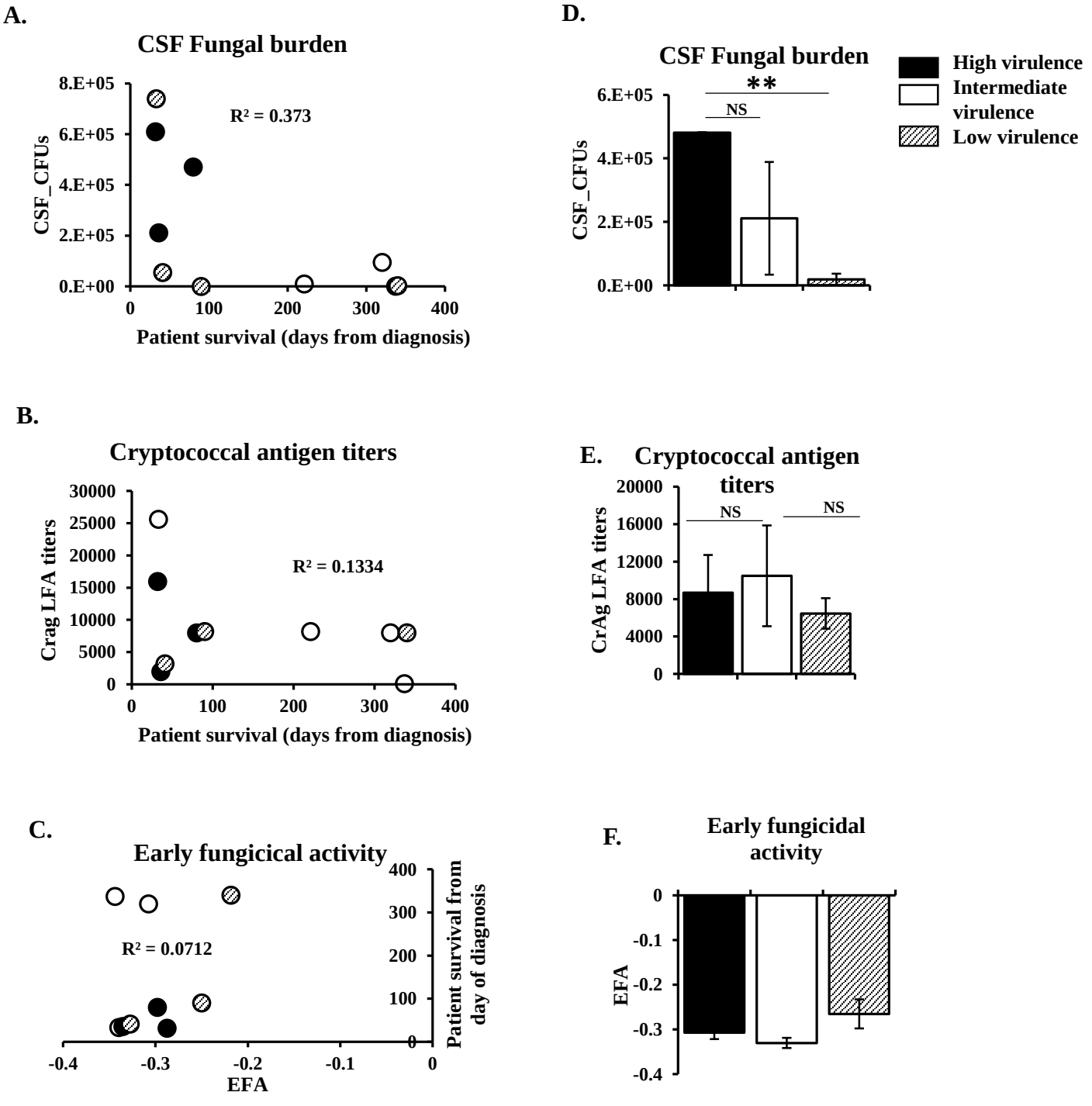
		LOCATION OF *CRYPTOCOCCUS* CELLS					IMMUNE RESPONSE		
	Strain	**Meninges**	**Cerebrum**	**Cerebellum**	**Hippo/thalamus**	**Granuloma**	**PMNs**	**Macrophages /Dendritic cells**	**Necrosis**
High virulence	**SACl012-1**	-	-	-	-	-	-	-	-
	SACl012-4	-	-	-	-	-	-	-	-
	SACl012-2	+	++	-	-	+	++	-	++
	SACl012-3	-	++	-	++	-	-	-	++
	UgCl302-1	+	-	++	-	-	+	+	-
	UgCl302-2	-	-	-	-	-	-	-	-
	UgCl387-1	-	-	-	-	-	-	-	-
	UgCl387-2	-	+	-	-	-	-	-	-
	UgCl395-1	-	-	-	-	-	-	-	-
Intermediate virulence	**UgCl538-2**	-	-	-	-	-	+	-	-
	UgCl538-1	-	-	-	-	-	-	-	-
	MbCl006-1	-	-	-	-	-			
	MbCl006-2	++	+++	++	-		-	-	++
	SACl059-1	+++	+++	+++	+	+++	+	-	+
	UgCl425-1	+++	++	+	+		-	-	-
	UgCl425-2	+++	+++	++	+++	+++	-	-	+++
Low virulence	**UgCl223-1**	+++	++	++	++	-	-	-	+++
	UgCl223-2	-	-	-	-	-	-	-	-

Presence of *Cryptococcus*: - none, + scattered, ++ multifocal, +++ confluent

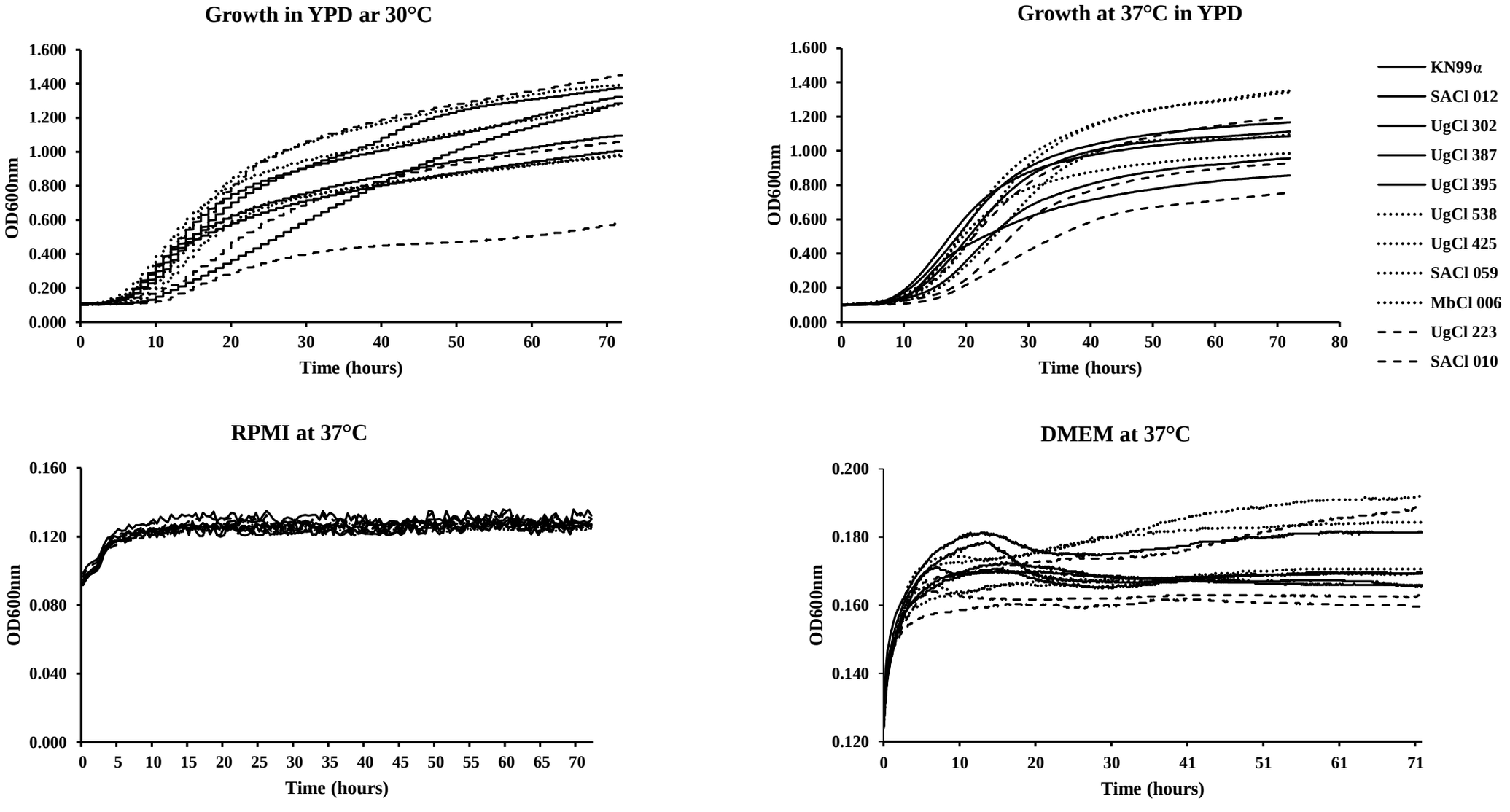
PMN: polymorphonuclear cells



Supplemental Figure 1. Human mortality is not determined by patient parameters. Clinical outcome was compared to baseline CM patient characteristics including CD4+ cells (A, D), white blood cell counts in the CSF (B, E), and HIV viral load (C, F). Graph A-C show individual patient parameters plotted against survival from diagnosis. Graphs D-F show patient parameters when classified by degree of virulence in the mouse model of cryptococcosis. Strains were classified in three groups: high (filled), intermediate (empty) and low virulence (striped) strains. Error bars represent standard error of the mean. CD4 cells: CD4+ T helper cells, CM: cryptococcal meningitis, CSF: cerebrospinal fluid, HIV: human immunodefiency virus, RNA: ribonucleic acid, WBC: white blood cells.

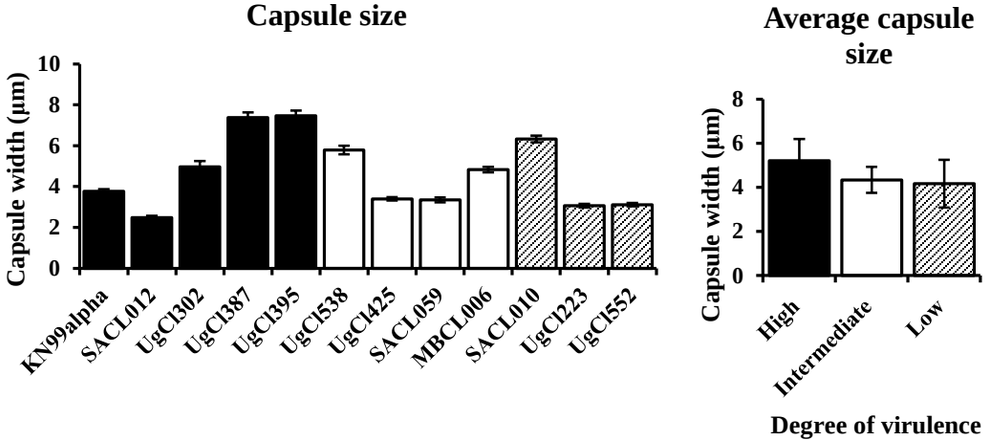


Supplemental Figure 2. Human mortality is partly determined by fungal parameters. Clinical outcome was compared to fungal parameters including CSF-fungal burden determined by colony forming units (CFUs) (A, D), cryptococcal antigen titers in the CSF (B, E) and rate of fungal clearance determined by the early fungicidal activity (EFA) (C, F). Graphs A-C show individual patient parameters plotted against survival from diagnosis. Graphs D-F show patient parameters when classified by degree of virulence in the mouse model of cryptococcosis. Strains were classified in three groups: high (filled), intermediate (empty) and low virulence (striped). Error bars represent standard error of the mean. CFU: colony forming unit, CrAg LFA: cryptococcal antigen lateral flow assay, NS: not statistically significant. ** $p < 0.01$

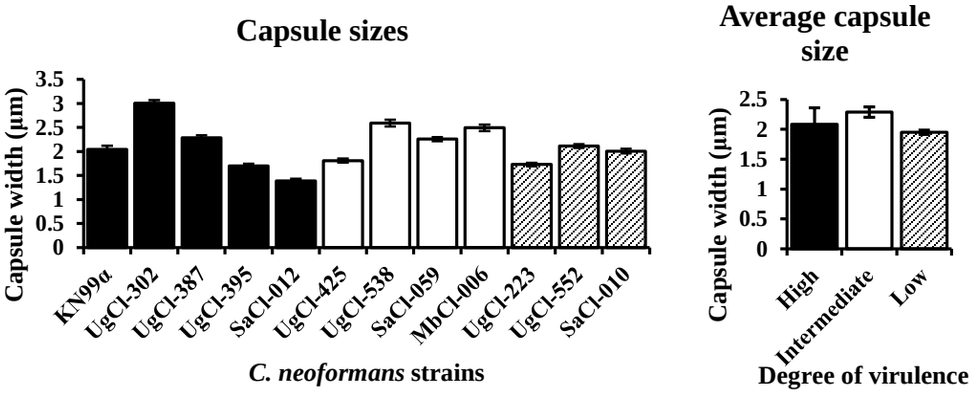


Supplemental Figure 3. Absolute growth. Eleven *C. neoformans* clinical strains and the wild type control (KN99α) were grown overnight in YPD broth at 30°C, washed with PBS, and counted with a hemocytometer. 5 X 10^3 yeast cells were transferred into 96-well plates containing 200 µl of either YPD (A, B), RPMI (C) or DMEM and grown at 30 or 37°C. Absorbances (OD600nm) were read during a 72 h incubation. Straight lines: high virulence strains, dotted lines: strains with intermediate virulence, dashed lines; low virulence strains. OD: optical density, DMEM: Dulbecco's Modified Eagle's Medium , RPMI: Roswell Park Memorial Institute medium, YPD: yeast extract-peptone-dextrose.

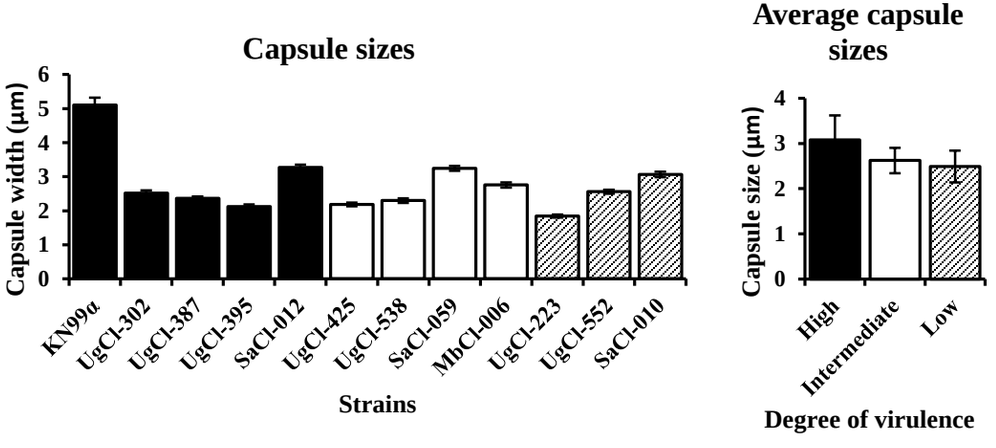
A. Capsule formation in DMEM+FCS



B. Capsule formation in Minimal Media



C. Capsule formation in PBS+FCS



Supplemental Figure 4. *In vitro* capsule formation. A *C. neoformans* wild type control strain and eleven clinical strains were induced to form capsule *in vitro* under three different conditions. **A)** Capsule induction in DMEM and serum: yeast cells were grown overnight in YPD, washed and then transferred into DMEM supplemented with FCS and incubated at 37°C, 5% CO_2 for 5 days. **B)** Capsule induction in Minimal media: yeast cells were grown 22 h at 30°C in YPD, washed and resuspended in minimal media at a final concentration of 1 x 10^6 cells/ml and incubated at 30°C with shaking (800rpm) in a thermomixer for 48 hours. **C)** Capsule induction in PBS and serum: yeast cells were grown overnight in YNB, washed and then transferred into PBS supplemented with FCS and incubated at 37°C, 5% CO2 for 48 hours. After incubation, yeast cells were fixed with 3.7% formaldehyde and observed under the microscope. The capsule width was defined as the difference between the diameter of the whole yeast cell (cell body and capsule) and the cell body diameter (no capsule) divided by 2. DMEM: Dulbecco's Modified Eagle's Medium , FCS: Fetal calf serum, PBS: Phosphate buffered saline, YNB: yeast nitrogen base medium, YPD: yeast extract-peptone-dextrose.