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Role of Quantitative CSF Microscopy to Predict Culture Status and Outcome in HIV-Associated Cryptococcal Meningitis in a Brazilian Cohort

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Abstract

Objectives—To evaluate clinical, laboratory, and quantitative cerebrospinal fluid (CSF) cryptococcal cell counts for associations with in-hospital outcomes of HIV-infected patients with cryptococcal meningitis.

Design—Retrospective study.

Methods—98 HIV-infected adult patients with CSF culture-proven cryptococcal meningitis admitted between January 2006 and June 2008 at a referral center in Sao Paulo, Brazil.

Results—Cryptococcal meningitis was the first AIDS-defining illness in 69% of whom 97% (95/98) had known prior HIV-infection. The median CD4+ T cell count was 39 cells/mcL (IQR: 17–87 cells/mcL). Prior antiretroviral therapy (ART) was reported in 50%. Failure to sterilize the CSF by 7–14 days was associated with baseline fungal burden of ≥10 yeasts/mcL by quantitative CSF microscopy (OR=15.3, 95% CI: 4.1–56.7;*P*<.001) and positive blood cultures (OR=11.5, 95% CI:1.2–109;*P*=.034). At 7–14 days, ≥10 yeasts/mcL CSF was associated with positive CSF cultures in 98% vs. 36% when <10 yeasts/mcL CSF (*P*<.001). In-hospital mortality was 30% and associated with symptoms duration for >14 days, altered mental status (*P*<.001), CSF WBC counts <5 cells/mcL (*P*=.027), intracranial hypertension (*P*=.011), viral loads >50,000 copies/mL (*P*=.036), ≥10 yeasts/mcL CSF at 7–14 days (*P*=.038), and intracranial pressure >50 cmH_20 at 7–14 days (*P*=.007).

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Conclusion—Most patients were aware of their HIV-status. Fungal burden of ≥10 yeasts/mcL by quantitative CSF microscopy predicted current CSF culture status and may be useful to customize the induction therapy. High uncontrolled intracranial pressure was associated with mortality.

Introduction

Cryptococcal meningitis causes significant overall mortality among HIV-infected patients from low and middle-income regions (Satishchandra et al., 2000; Harrison, 2009; Ganiem et al., 2009; Prado et al., 2009; Jarvis et al., 2010). Most published studies have been reported from sub-Saharan Africa, South Asia, and Southeast Asia, but data are scarce from South America (Park et al., 2009).

AIDS-associated cryptococcosis has decreased less dramatically in Brazil (Guimarães, 2000; Pappalardo et al., 2003), a middle-income country with universal access to antiretroviral therapy (ART) (Greco et al., 2007), when compared to developed countries (Mirza et al., 2003; Jarvis et al., 2007). Cryptococcal meningitis continues to be a common complication in Brazil (Leimann et al., 2009), representing the primary cause of opportunistic meningitis and the second most frequent neurologic opportunistic infection in HIV-infected patients (Oliveira et al., 2006; Vidal et al., 2008).

In this study, we report clinical and laboratory characteristics associated with clinical outcomes among HIV-infected patients with cryptococcal meningitis at a referral center in Sao Paulo, Brazil.

Patients and Methods

We retrospectively studied HIV-infected adult patients with a first diagnosis of cryptococcal meningitis admitted between January 2006 and June 2008 at the Emilio Ribas Institute of Infectious Diseases, Sao Paulo, Brazil. This hospital is a 250-bed tertiary teaching center and is the main public referral institution for HIV-infected patients in the Sao Paulo State. The institute serves primarily a population with low socioeconomic status. In these patients, HIV infection was diagnosed by enzyme-linked immunosorbent assay (ELISA) and confirmed by western blot. The diagnosis of cryptococcal meningitis was based on *Cryptococcus neoformans* cultured from cerebrospinal fluid (CSF). Quantitative CSF cultures were not performed, but quantitative CSF yeast cell counts were performed by direct microscopic exam with a Fuchs-Rosenthal cell counting chamber, quantifying how many yeast cells/mcL of CSF were present (the protocol is showed in Table 1).

At the Emilio Ribas Institute of Infectious Diseases, routine clinical care consists typically of all HIV-infected patients receiving a brain computed tomography (CT) scan prior to lumbar puncture. After diagnosis, the patients usually received induction therapy with amphotericin B deoxycholate (0.7 mg/kg/day) for 4–6 weeks. If necessary, more prolonged induction therapy was used. Consolidation therapy was with oral fluconazole 400–800mg per day for at least 4–6 weeks.

Disseminated cryptococcosis was defined as the involvement of ≥2 noncontiguous sites. Increased ICP was defined as opening CSF pressure >20 cm H_2O, measured via lumbar puncture (LP). In cases of opening ICP ≥25 cm H_2O, the routine practice included repeat therapeutic LPs daily until the CSF opening pressure and symptoms were stabilized. Neurosurgery was pursued for refractory elevated ICP if therapeutic LPs failed to normalize pressures, typically after >14 days. Routine CSF diagnostic studies including culture were performed weekly while hospitalized. Patients were routinely discharged after two sterile CSF cultures.

For this retrospective cohort study, we extracted demographic, clinical, and laboratory data from medical records using a standardized questionnaire. CSF findings were classified in two groups: 'diagnostic CSF' collected at initial hospital admission and 'follow up CSF' collected 7–14 days after admission. Analysis was primarily descriptive. The primary analysis focused on risk factors associated with in-hospital mortality. Statistical testing between groups (survivors vs. non-survivors) was assessed by Mann-Whitney U test for continuous variables with distributions presented as median and interquartile range (IQR). Fischer's exact test was used for categorical variables. Factors associated with mortality in a univariate model with $P<0.2$ were considered as potential covariates in a multivariate logistic regression model (SPSS 18.0, IBM, Chicago, IL, USA). All *P*-values were two-sided. Variables with $P \leq 0.05$ remained in the multivariate model with risk presented as an adjusted odds ratio (OR). The study was approved by the ethical and scientific boards of the Emilio Ribas Institute of Infectious Diseases.

Results

Demographic, clinical and laboratory characteristics

During the 30-month study period, 113 patients had CSF cultures positive for *C. neoformans*. Eight (7%) records were unavailable, and seven (6%) cases did not meet the inclusion criteria (six HIV-negative, and one child). Of the 98 adults included in the study, 76 (78%) were male with a median age of 38 (range: 18–56) years.

HIV diagnoses were most often not new with 97% (95/98) having a prior HIV diagnosis, 31% (30/98) having previous AIDS-defining disease, and 50% (46/98) having prior ART use, though ART adherence was unknown. The time between HIV diagnosis and cryptococcal meningitis was a median of 84 months (IQR: 12–120 months). The median CD4+ T cell count (n=85) was 36 cells/mcL (IQR: 17–87 cells/mcL). Sixty five percent of patients (n=55) had CD4+ T cell count ≤50 cells/mcL and 14% (n=12) had 51–100 cells/mcL. However, cryptococcosis also occurred at higher absolute CD4+ T cell counts: 12% (n=10) had 101–200 cells/mcL and 9% (n=8) had >200 cells/mcL. CD4+ T cell counts were similar among patients with and without prior ART use with a median of 30 cells/mcL (IQR: 16–72 cells/mcL) in ART-naïve vs. 39 cells/mcL (IQR: 19–96 cells/mcL) with prior ART use ($P=.42$). Of 34 persons prescribed ART who had a viral load measured, only 6 (18%) had HIV-1 viral loads <400 copies/mL of whom only 2 had <50 copies/mL. Data were unavailable on duration of ART use and ART adherence.

Presenting symptoms were typical for cryptococcal meningitis. The most frequent symptoms were headache in 91% (87/96), nausea/vomiting in 56% (54/96), and fever in 55% (53/96). Altered mental status was observed in 31% (30/96) cases with a GCS<15, but coma was reported in only 3% (3/96) cases. Meningeal signs were documented in 12% (11/93) and seizures present in 17% (16/96). Neurologic symptoms were present at admission for a median of 14 days (IQR: 5–26 days). At admission, increased ICP was present in 54 (55%) cases. The median ICP was 31 cm H_2O (IQR: 20 – 48 cm H_2O). Serum cryptococcal antigen detection was positive in 37 (90%) of 41 patients tested.

Disseminated disease in addition to meningitis was reported in 52% (49/94) of cases: bloodstream fungaemia in 28% (n=26), pulmonary infection in 11% (n=10), bone marrow in 4% (n=4), and dermatologic manifestations in 2% (n=2). Concomitant pulmonary and bloodstream infection were observed in 6% (n=6), and concomitant pulmonary, bloodstream, skin and bone marrow in 1 person. Brain CT abnormalities were observed in 26 (31%) of 83 persons who received a CT and were: brain atrophy (n=20), pseudocysts (n=10), cryptococcoma(s) (n=7), and diffuse brain edema (n=2).

Quantitative CSF Microscopy—Quantitative CSF yeast counts were routinely performed. The diagnostic CSF specimen collected at admission had a median of 71 yeast/mcL of CSF (IQR: 3–512; range: 0 – 8160) and declined during amphotericin therapy with follow up CSF collected 7–14 days having a median of 11 yeast/mcL (IQR: 0 to 108, max: 10720, $P<.0001$) with 55% of follow up CSF remaining culture-positive (Figure 1). On the follow up CSF, survivors had fewer yeast than non-survivors (median 6 vs. 81 yeast/mcL, $P=.035$) at 7–14 days. By three weeks, the quantitative yeast burden had decreased (median 1.0 yeast/mcL, IQR: 0 to 11, max: 4,566, $P<.001$).

We analyzed variables associated with lack of CSF culture sterility at 7–14 days. In univariate analysis, CD4 cell count <100 cells/mcL ($P=.008$), disseminated disease ($P=.003$), fungemia ($P=.003$), baseline fungal burden ≥ 10 yeast/mcL of CSF ($P<.001$), and CSF WBCs ≤25 cells/mcL ($P=.022$) were statistically associated with a positive follow up CSF culture. In a multivariate model, only baseline CSF fungal burden ≥10 yeast/mcL by quantitative microscopy (OR=15.3, 95% CI: 4.1–56.8; $P<.001$) and baseline fungemia (OR=11.5, 95% CI: 1.2–109; $P=.034$) remained associated with a positive follow up CSF culture at 7–14 day of amphotericin therapy. As all subjects received similar antifungal treatment, the burden of infection was the predominant factor affecting the timing of culture sterility.

The quantitative microscopy yeast count was useful in predicting the week two culture-status. Persons with quantitative CSF yeast counts <10/mcL at 7–14 days had positive CSF cultures in 37.5% (12/32) as compared to 98% (41/42) positive CSF cultures when ≥10 yeasts/mcL ($P<.001$). Similarly, among those with LPs performed between 15–21 days, quantitative microscopy of <5 yeasts/mcL CSF had 16% (6/37) CSF culture positivity vs. 57% (8/14) CSF culture positivity when CSF yeasts were ≥5 yeasts/mcL CSF ($P=.011$).

Intracranial Pressure—Management of increased ICP was not always optimal and/or there was not documentation of follow up therapeutic LPs. Of 38 patients with known initial increased ICP of >25 cmH_2O, 68% (26/38) had documentation of receiving a follow up therapeutic LP. Of 34 patients who did not have an initial opening pressure documented, only 26% (9/34) had therapeutic LPs similar to the 26% (5/19) incidence when the ICP was initially measured as <25 cmH_2O. Seven persons had missing documentation on therapeutic LPs. We analyzed variables associated persistently increased ICP at 7–14 days of treatment among 54 persons with a second LP with an opening pressure measured. Only CSF fungal burden ≥10 yeast/mcL collected between 7–14 days was associated with persistently elevated ICP >20 cmH_2O between 7–14 days (OR=6.5, 95% CI: 1.7–24.4; $P=.006$).

Neurosurgery was performed in 18% (18/94). In 17 patients, the surgical indication was failure to control increased ICP after repeated therapeutic lumbar punctures, typically after ≥2 week of daily LPs. Interventions included: ventriculoperitoneal shunt (n=9), external lumbar drainage (n=6), external ventricular drainage (n=2), and lumbar peritoneal shunt (n=1). One of these patients first underwent external lumbar drainage which was then later converted to a ventriculoperitoneal shunt. In the one additional case without refractory increased ICP, the surgical indication was hydrocephalus at admission. Median time from admission to neurosurgery was 25 days (IQR: 16–42 days). Survival was 67% (6/9) in ventriculoperitoneal shunts and 17% (1/5) in external lumbar drains.

Outcomes and characteristics associated with mortality

The in-hospital case-fatality rate was 30% (n=29). The attribute cause of death was cryptococcal meningitis in 52% of deaths (n=15) and other nosocomial infections in 48% (n=14). Among patients undergoing neurosurgery, the case-fatality rate was 56% (10/18).

Demographic, clinical, and laboratory features in survivors and nonsurvivors patients are shown in Table 2.

We identified a number of demographic features associated with all-cause mortality (Table 3). Most notably, independent risk factors for mortality included: antecedent symptom duration >14 days, abnormal mental status, HIV-1 viral load >50,000 copies/mL, normal CSF WBC counts <5 cells/mcL, increased ICP, persistently increased ICP between 7–14 days.

Of the 89 persons who had a second LP, death was associated with a quantitative fungal burden ≥10 yeast/mcL on CSF collected 7–14 days (OR=6.9, 95% CI: 1.1–43.0; *P*=.038) and an opening pressure of >50 cmH_2O (OR=14.6, 95% CI: 1.7–124, *P*=.014) as adjusted for baseline independent mortality risk factors listed above. For increased ICP, only opening pressures >50 cmH_2O at 7–14 days were associated with increased mortality of 50% (7/14), as persons with ICP between 20–49 cmH_2O had minimal increased mortality of 21% (5/24) (adjusted OR=1.1, *P*=.96) compared with normal ICP <20 cmH_2O mortality of 12.5% (2/16). Of the 36 persons who did not have an opening pressure measured on their second LP, they also trended toward increased mortality of 25% (9/36) (adjusted OR=1.8, *P*=.51) as compared to the 16 persons with normal ICP <20 cmH_2O, suggesting that a subset likely had markedly increased ICP. Thus while, the initial baseline fungal burden was not associated with in-hospital mortality, the follow up burden during week two was associated with mortality as was ongoing uncontrolled ICP of >50 cmH_2O during week two.

Discussion

This study revealed three important clinical findings. First, quantitative CSF microscopy was a simple and useful tool which beyond 7 days was associated with CSF culture status and survival. Second, initial ICP was not associated with outcome; however uncontrolled ICP of >50 cm H_2O after >7 days was associated with high mortality (≥50%). Third, we found a continued high case-fatality rate among Brazilian HIV-infected patients with cryptococcal meningitis. At admission, most patients were aware of their HIV-status, although cryptococcal meningitis was their first AIDS-defining condition. The majority of patients presented with features of severe disease including: abnormal mental status, increased ICP, disseminated disease, neurologic manifestations, and severe immunosuppression.

First, this study systematically utilized quantitative CSF microscopy to estimate fungal burden. This is a simple laboratory technique which can be processed while measuring a CSF WBC differential. Although quantitative CSF cultures can also identify fungal burden and the rate of clearance (Bicanic et al., 2009a; Bicanic et al., 2009b; Bicanic et al., 2011), quantitative cultures are not often used in routine practice, and there is an intrinsic delay in reporting. Thus, rapid and simple methods to estimate fungal burden remain clinically useful worldwide. In Brazil, several tertiary laboratories use the direct exam with the Fuchs-Rosenthal cell counting chamber to estimate the quantitative burden of infection. We evaluated several cut-offs (data not shown) but the best cutoff was ≥10 yeasts/mcL in CSF collected after 7 days of treatment which was independently associated with CSF culture positivity and all-cause mortality. A very useful clinical test was ≥10 yeasts/mcL in CSF at 7–14 days was associated with 98% CSF culture positivity. This cut-off is clinically useful to predict who should be targeted for prolonged induction amphotericin therapy or higher dose consolidation therapy starting at fungicidal doses of fluconazole at ≥800mg/day.

Second, only very high opening pressure in the CSF of >50 cm H_2O when collected ≥7days after admission were associated with mortality. These findings confirm the value of ICP

management during cryptococcal meningitis treatment in that lack of ICP control is associated with mortality. Similar findings have been reported previously in three African locations (Bicanic et al., 2009a; Bicanic et al., 2009b, Bicanic et al., 2011; Longley et al., 2008, Kambugu et al., 2008). Bicanic *et al.* reported a significant association between day 14 CSF fungal burden and day 14 opening pressure, suggesting that improved clearance of infection may help prevent persistence of raised ICP (Bicanic et al., 2009a). We also identified a significant association between increased ICP and ≥10 yeasts/mcL in CSF collected 7 to 14 days after admission.

Third, the present study reported an in-hospital mortality of 30%, despite being performed in a referral center with a reasonable infrastructure. There is scarce information about case-fatality rate of HIV-infected patients with cryptococcal meningitis from South America in routine practice. In unselected hospital-based studies performed in Brazil and Argentina, case-fatality rates have ranged from 31.5% to 62.5% (Metta et al., 2002; Pasqualotto et al., 2004; Moreira et al., 2006; Pappalardo et al., 2007; Mónaco et al., 2008; Lindenberg et al., 2008; Mora et al, 2011). Taken together, these studies confirm that acute mortality of cryptococcal meningitis remains unacceptably high in South America despite the administration of amphotericin B yet with frequent non-optimal measures of ICP control.

Limitations

Limitations include problems with a retrospective study including the absence of a standardized history, lab information, and neurologic examination as well as long-term follow up data. ,Although this is a large series of AIDS-associated cryptococcal meningitis of a single institution, we these results are representative of outcomes in routine practice in Brazil with the available sample size allowing for the detection of the most significant clinical associations with mortality.

In conclusion, AIDS-associated cryptococcal meningitis remains an important cause of mortality in Brazil, despite the increasing ART availability. We report that quantitative CSF microscopy is a useful intervention to predict CSF culture status and thereby potentially to tailor anti-fungal therapy to an individual patient. We believe that quantitative CSF microcopy is a simple tool which is broadly implementable. Future challenges in our setting include: optimizing adjunct antifungal treatment (e.g. addition of 5-flucytosine or fluconazole ≥800mg/day to amphotericin-based therapy), evaluating the role of alternative approaches (e.g. immunotherapy such as adjunct interferon-gamma), evaluating standardized ICP management, and finally determining the most efficient public health strategy to avoid severe immunosuppression in HIV-infection patients who are aware of their HIV-status.

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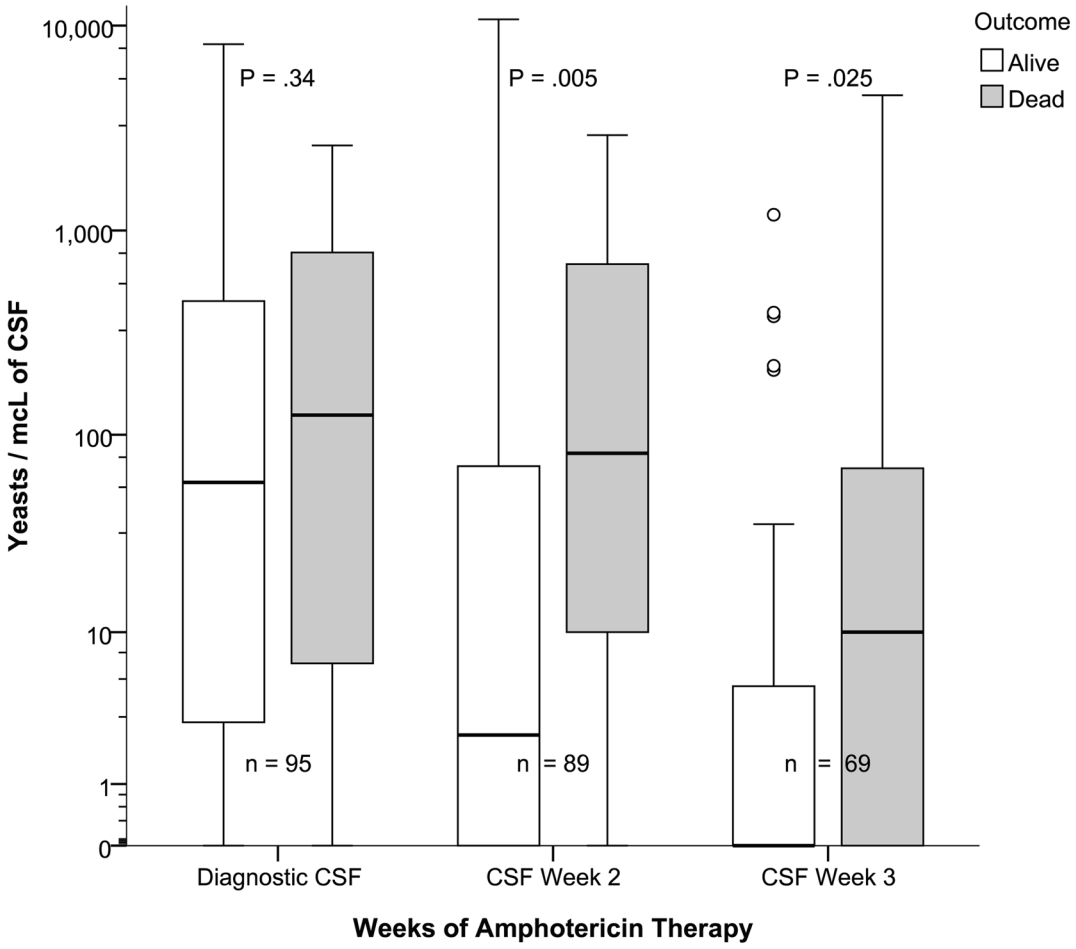


Figure 1.
Temporal change in quantitative cerebrospinal fluid yeast counts.

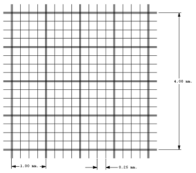
Table 1

The Fuchs-Rosenthal counting chamber can be used for cerebrospinal fluid (CSF) cell counts. This is the normal cell counting chamber that is often used to measure WBC total and differential counts. We adapted this method to quantitatively count yeasts in patients with cryptococcal meningitis.

1) PREPARING THE SAMPLE

The CSF collected in a sterile test tube should contain no visible trace of blood. To place a cover slide in the chamber. After carefully manual homogenization the CSF is filled by capillary attraction into the chamber using a pipette. The CSF will be filled into the chamber without having to be stained first. Care is to be given to make sure that just enough CSF is drawn up with no air bubbles and that the CSF fluid does not spill over the edges of the chamber. After a sedimentation period of approximately one minute, the counting of the yeasts can begin.

2) CALCULATING THE YEAST DENSITY



A) SETTING ONE: LOW BURDEN

Computing the volume

"A" = total area has 16 large squares sub-divided in 256 small squares

"AR" = Area of each small square: 0.0625 mm^2

"P" = Depth of each one of the small square: 0.2 mm

"VR" = Volume of each small square:

Calculating formula:

$$\text{"VR"} = P(0.200\ \text{mm}) \times \text{"AR"}(0.0625\ \text{mm}^2) = 0.0125\ \text{mm}^3$$

"VT" = Total volume of the chamber:

Calculating formula:

$$\text{"VT"} = VR(0.0125\text{mm}^3) \times \text{"A"}(256\ \text{small squares}) = 3.2\ \text{mm}^3$$

Calculating the number of yeasts (yeasts/mm^3)

Count all the chamber (256 small squares) and divide by "VT" (3.2 mm^3) = yeasts/mm^3 or mcL

B) SETTING TWO: HIGH BURDEN

If there are more than 10 yeasts in each small square, to do a mean (M) in 10 small squares and to multiply by a correction factor (F). The result will be expressed in yeasts/mm^3or mL

$$\text{Correction factor (F)} = \text{"number of small squares"}(256)/\text{"VT"}\ (3.2) = 80$$

Example: M=19, thereby: 19X80=1520 yeasts/mm^3or mcL

Table 2

Demographic, clinical and laboratory features in survivors and non-survivors in 98 HIV-infected patients with cryptococcal meningitis.

Variable, median (IQR)	Survivors n=69	Non-survivors n=29	*P* - value
Age years	39 (32.5–44)	38 (35– 42.5)	0.63
Gender (male, %)	52 (75%)	24 (83%)	0.30
Previous HIV diagnosis, n (%)	66 (96%)	29 (100%)	0.55
Previous AIDS-defining illness, n (%)	22 (35%)	8(32%)	0.81
Prior ART use, n (%)	32 (48%)	14 (48%)	0.99
Duration of HIV diagnosis in months	96 (25.5–120)	60 (15–120)	0.52
Symptoms duration in days	14 (5–20)	16.5 (7.25–30)	**0.030**
Disseminated disease, n (%)	35 (54%)	14 (52%)	0.99
Blood culture positivity, n (%)	18 (27%)	8 (30%)	0.80
Increased ICP, n (%), n=89	33 (51%)	21 (87%)	**0.003**
Abnormal mental status	14 (20%)	16 (55%)	**0.001**
Seizures, n (%)	7 (10%)	9 (31%)	**0.012**
Need for CSF shunt / drainage, n (%)	8 (12%)	10 (34.5%)	**0.011**
HIV RNA $\log_{10}$ copies/mL	4.1 (3.2–5.3)	5.5 (5.0–5.7)	**0.004**
CD4 count cells/mcL, n=85	38 (21–106)	44 (16 –110)	0.97
Hemoglobin g/dL	12.0 (10.6 – 13.5)	11.9 (10.0– 13.0)	0.60
Opening pressure of CSF, cm H_2O			
CSF at admission, n=43,16	30 (18–41)	36 (22 – 71)	0.23
CSF at 7–14 days, n-40, 14	21 (13.5– 38)	48 (22 – 75)	**0.027**
CSF at >14 days, n=24, 8	17.5 (10–25)	28.5 (22–58)	**0.013**
CSF quantative yeasts/mcL count			
CSF at admission, n=68, 27	58.5 (3 – 453)	125 (8 – 782)	0.34
CSF at 7–14 days, n=66, 23	3 (0 – 70)	81 (10 – 690)	**0.005**
CSF at 15–21 days, n=50, 19	0 (0 – 5)	10 (0 – 90)	**0.025**

ART = antiretroviral therapy; CSF = cerebrospinal fluid. ICP = intracranial pressure. Non-parametric data are presented as median and interquartile range (IQR). Categorical and nominal data are presented as n(%). Statistical testing is by Fisher's exact test for categorical variables, Mann-Whitney U testing for non-parametric data.

Table 3

Multivariate risk factors for all-cause mortality in 98 HIV-infected patients with cryptococcal meningitis.

Variable	Adjusted Odds Ratio	95% CI	*P*-value
Abnormal mental status	18.0	3.8 – 84	< **0.001**
Symptoms duration			
<14 days	1.0	Ref	Ref
15–28 days	3.9	0.7 – 22.5	0.12
>28 days	10.8	2.0 – 57.3	**0.005**
CSF WBC count			
≤ 5 cells/mcL	4.6	1.1 – 18.9	**0.027**
6–25 cells/mcL	2.3	0.3 – 20.1	0.40
>25 cells/mcL	1.0	Ref	Ref
CNS shunt placement / drainage	2.8	0.6 – 12.8	0.19
Increased intracranial pressure	15.0	2.1 –107	**0.007**
Viral load > 50,000 copies/mL	5.8	1.1 – 30	**0.037**
At day 7–14 lumbar puncture [1]			
CSF quantitative yeast count ≥10/mcL (n=89)	6.9	1.1 – 43.0	**0.038**
Opening pressure ≥50 cm H_20 (n=14)	14.6	1.7 – 124	**0.014**
Opening pressure not documented (n=35)	5.7	0.5 – 63.4	0.154
Opening pressure <50 cm H_20 (n=40)	1.0	Ref	Ref

[1] Day 7–14 lumbar puncture variables are adjusted for baseline predictors but not vice versa.