Mortality due to *Cryptococcus neoformans* and *Cryptococcus gattii* in low-income settings: an autopsy study

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Cryptococcosis is a major opportunistic infection and is one of the leading causes of death in adults living with HIV in sub-Saharan Africa. Recent estimates indicate that more than 130,000 people may die annually of cryptococcal meningitis in this region. Although complete diagnostic autopsy (CDA) is considered the gold standard for determining the cause of death, it is seldom performed in low income settings. In this study, a CDA was performed in 284 deceased patients from Mozambique (n = 223) and Brazil (n = 61). In depth histopathological and microbiological analyses were carried out in all cases dying of cryptococcosis. We determined the cryptococcal species, the molecular and sero-mating types and antifungal susceptibility. We also described the organs affected and reviewed the clinical presentation and patient management. Among the 284 cases included, 17 fatal cryptococcal infections were diagnosed. *Cryptococcus* was responsible for 16 deaths among the 163 HIV-positive patients (10%; 95%CI: 6–15%), including four maternal deaths. One third of the cases corresponded to *C. gattii* (VGI and VGIV molecular types, Bα and Cα strains) and the remaining infections typed were caused by *C. neoformans var. Grubii* (all VNI and Aα strains). The level of pre-mortem clinical suspicion was low (7/17, 41%), and 7/17 patients (41%) died within the first 72 hours of admission. Cryptococcosis was responsible for a significant proportion of AIDS-related mortality. The clinical diagnosis and patient management were inadequate, supporting the need for cryptococcal screening for early detection of the disease. This is the first report of the presence of *C. gattii* infection in Mozambique.

Cryptococcosis is the leading cause of meningitis in adults living with HIV in sub-Saharan Africa. In 2008, the number of cryptococcal meningitis (CM) cases in this region was estimated to be 720,000 (range 144,000–1.3

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partum, post-partum or within 42 days of termination of pregnancy15), and 54 were children from 1 month to
patients in the two study sites. Two hundred twenty-three cases were recruited from Mozambique: 169 were
Collaborations, and the Singapore Statement on Research Integrity.

From November 2013 to March 2015, complete diagnostic autopsies (CDAs) were performed in 284 deceased
patients in the two study sites. Two hundred twenty-three cases were recruited from Mozambique: 169 were
adults over 15 years of age (112 women, 57 of whom were maternal deaths, i.e., women dying during pregnancy,
partum, post-partum or within 42 days of termination of pregnancy19), and 54 were children from 1 month to
15 years of age (37 males and 17 females). Sixty-one cases were recruited from Brazil: 59 adults (38 males and
21 females, including one maternal death) and two children. A description of the study design and inclusion
criteria for the cases has been published elsewhere16-18. All cases fulfilling the inclusion criteria were included
in the study; these were (1) a CDA requested by the clinician as part of the medical evaluation of the patient and;
(2) a verbal informed consent to perform the autopsy given by the relatives. Traumatic deaths were excluded. A
member of the study staff was tasked with liaising with the families in cases of deaths occurring in the pediatric
department, but only after the clinicians had asked for consent for postmortem examination17. All the clinical
records available regarding admission preceding death of each patient were reviewed and the clinical data were
collected in a standardized manner.

Autopsy procedures and determination of the cause of death. The autopsy procedures and a
description of the pathological and microbiological methods used have been reported elsewhere19-22 and for
briefly, samples of blood, cerebrospinal fluid (CSF), bone marrow and key organs such as the liver, lungs, CNS,
spleen and kidneys, as well as uterus in all women of reproductive age were collected for histopathological and
microbiological analysis. The microbiological methods included universal screening for relevant pathogens (e.g.
HIV, viral hepatitis, tuberculosis, malaria) and bacterial/fungal culture of autopsy samples, targeted screening
depending on the patient’s condition (i.e. screening for Cryptococcus, Toxoplasma gondii and Pneumocystis
jiroveci in HIV-infected cases), and additional specific testing according to the histopathological findings.
Following the analysis of the CDA samples, a panel composed of a pathologist, a microbiologist, and a clinician
with expertise in infectious diseases and epidemiology evaluated all the data (including the clinical information)
and assigned the final cause of death.

Laboratory methods. All cases in which the cause of death was assigned to a cryptococcal infection were
further characterized. The histological evaluation included periodic acid–Schiff staining (PAS) of the samples of
both lungs, CNS, bone marrow, spleen, liver, kidney and uterus, as well as Grocott-Gomori methenamine sil-
ver staining in all CNS samples. Microbiological evaluation included a specific real time PCR for Cryptococcus
spp.21, which was performed in the samples of both lungs, CNS, bone marrow, spleen, liver and uterus. PCR cycle
threshold values >38 were considered negative. Plasma and CSF samples were tested by both real time PCR and the Cryptococcus antigen (CrAg) lateral flow assay (LFA) (IMMY Inc., Norman, Oklahoma). Discrimination between *C. neoformans* var. *grubii*, *C. neoformans* var. *neofor- mans*, and *C. gattii* was achieved by amplification of the rRNA intergenic spacer (IGS) region, followed by forward and reverse Sanger sequencing of the amplicons. Identities of the cryptococcal species were assigned based on a >98% match to the IGS sequence of a Cryptococcus reference strain (*C. gattii* CBS 6289, ATCC MYA-4561, CBS 6955, ATCC MYA-4563; *C. neoformans* var. *grubi*- ATCC MYA-4564, ATCC MYA-4565 and *C. neoformans* var. *neofor- mans*: ATCC MYA-4567) as described previously.22

Cryptococcal strains isolated from the cultures of the autopsy samples underwent a consensus multi-locus sequence typing scheme for *C. neoformans* and *C. gattii*.23 In addition, the sero-mating type of these strains was determined by multiplex PCR as described previously.24,25 An anti-fungogram was performed for each strain using the Sensititre® YeastOne® susceptibility plate (TREK Diagnostic Systems, Thermo Fisher Scientific, Oakwood Village, USA).

**Statistical methods.** Descriptive analysis was performed using univariate statistics with means and standard deviations for continuous variables and frequency distributions for categorical variables. All analyses, data manipulation, and implementations were done using Stata MP version 15 (Stata, College Station, TX, USA).

**Results**

**Mortality due to cryptococcal infections.** Among the 284 deceased patients included in this study, 163 (57%) tested positive for HIV, and a total of 17 (6%) fatal cryptococcal infections were confirmed in the CDA. All the deaths except one occurred in HIV-infected cases. Among the HIV-infected cases, cryptococcosis was responsible for 16 deaths (10%; 95% confidence interval [CI]: 6–15%). Among the Mozambican adults, 109 out of 169 (64%) were HIV-infected, and 11 died of fatal cryptococcal infection (10%). Four of these cases were maternal deaths (three pregnant women and one in the puerperal period), accounting for 11% of the 36 maternal deaths occurring in HIV-infected women. None of the 17 HIV-positive children over one month of age had cryptococcal infection. Thirty-seven out of the 61 (61%) patients from Brazil were HIV-infected, and Cryptococcus was responsible for five of these deaths (13%). The only death caused by *Cryptococcus* in an HIV-negative patient occurred in a six-year-old child (infected with *C. gattii*).

**Clinical presentation and management of fatal cryptococcal infections.** The demographic features, HIV status, clinical presentation and management of the 17 patients with fatal cryptococcal infection are summarized in Table 1. The median age was 34 years (range 6–44 years); 11 cases (65%) were men. In 13 out of the 16 (81%) HIV-infected patients, a positive HIV test result was reported in the clinical records and was apparently unknown by the clinician in the other 3 cases. Four out of the 16 (25%) HIV-infected patients were on ART, but the duration of ART was only reported in one case.

Headache was the most common symptom (13 patients, 76%), followed by fever and vomiting (eight cases each, 47%). Upon admission to hospital, eight patients (47%) were confused and/or agitated, two patients (12%) were lethargic, and another two (12%) were fully comatose. Meningeal signs were detected in seven patients (41%). The mean time from admission to death was 9.3 days (95%CI: 2.4–16.2). Cryptococcal infection was considered the first clinical diagnostic option in only 4/17 (23%) of the confirmed cases, whereas it was included in the differential diagnosis in eight patients (47%). Antifungal treatment (fluconazole or Amphotericin B) had been prescribed to seven patients but to only five of the clinically suspected cases. Seven of the patients (41%) died within 72 hours of admission, and 12 out of the 16 HIV-positive patients (75%) died within one week of admission.

The clinical records of the remaining 267 cases included in this study were reviewed for clinical diagnosis of cryptococcal infection. Among these, three HIV-infected patients were clinically diagnosed with cryptococcal meningitis, but no evidence of cryptococcal infection was found in the autopsy (the cause of death was identified as toxoplasmosis in two cases and tuberculosis in one case).

**Histopathological and microbiological analysis of the fatal cryptococcal infections.** Table 2 shows the final cause of death, the cryptococcal species, other associated diagnoses identified in the CDA, as well as the results of the PAS staining, *Cryptococcus* real time PCR and CrAg test in the different samples analysed. Identification of the cryptococcal species was successful in 15 out of the 17 cases. In two cases, the tissue samples provided insufficient DNA to perform IGS amplification and sequencing. Interestingly, among the 15 cases identified, five (33%) were *C. gattii* whereas the remaining cases (66.6%) were *C. neoformans* var. *grubii*.

Twelve of the 17 cases (70%) were diagnosed as disseminated infections and the remaining 5 (30%) as meningitis and/or meningoencephalitis. The CNS was affected in all 17 cases. The following were the most affected organs (PAS and/or PCR positive): the lungs (88%), spleen (76%), liver (71%), bone marrow (59%) and kidney (47%). In addition, in five out of six (83%) deceased women, the pathogen was detected in uterus samples, and in one case it was detected in the placenta. Cryptococcus was found in more than 5 different samples in 11 patients (65% of the cases). More organs were affected in *C. neoformans* var. *grubii* than in *C. gattii* infections (mean of 6.2 vs. 2.8 organs positive by PAS staining), but no specific histopathological differences were observed between the two *Cryptococcus* species. Molecular testing expanded the detection of *Cryptococcus* in 14 additional tissues that were negative for PAS staining. In contrast, two tissue samples were positive for PAS staining but negative by real time PCR. Periodic acid–Schiff staining and Grocott-Gomori-methenamine silver staining showed identical results. Representative histopathological images of different affected tissues are shown in Fig. 1. *Cryptococcus* was detected by PCR in CSF and plasma in 80% and 47% of the cases tested, respectively. All plasma and CSF samples tested for CrAg LFA were positive. Co-infections with other AIDS-related pathogens or AIDS-defining illnesses
| Case | Age (in years), sex and origin | Maternal death | HIV Serology | HIV Viral load (copies/mL) | Main symptoms | Pre-mortem Clinical Diagnoses* | Antimicrobial treatment received during illness | Antifungal treatment | Time from admission to death (days) | ART | ART comment |
|------|-----------------------------|----------------|--------------|---------------------------|---------------|-------------------------------|-----------------------------------------------|----------------------|-----------------------------------|------|-------------|
| 1    | 6: M; MOZ                   | NA             | Negative     | NA                        | Fever, seizure, headache, bilateral exophthalmos | Cerebral cryptococcosis,  | Penicillin, chloramphenicol, cephalosporin, quinolones, acyclovir | No | 52.3 | No |
| 2    | 30; F; MOZ                  | Yes            | Positive     | 28,600                    | Fever, vomits, headache, behavioral changes | HIV disease resulting in encephalopathy, unspecified HIV disease, pneumonia, organism unspecified | Co-trimoxazole, penicillin, cephalosporin | Fluconazole | 24.4 | Yes 72 months |
| 3    | 29; F; MOZ                  | No             | Positive     | >10,000**                 | Fever, cough, vomiting, seizure, headache, night sweats, hematemesis | Encephalitis, myelitis and encephalomyelitis, unspecified HIV disease | Cephalosporin, acyclovir | Fluconazole | 2.9 | NA |
| 4    | 34; M; MOZ                  | NA             | Positive     | 7,720                     | Fever, vomiting, headache, behavioral changes | Encephalitis, myelitis and encephalomyelitis | Cephalosporin | No | 1.1 | NA |
| 5    | 36; M; MOZ                  | NA             | Positive     | 1,180                     | Headache, exophthalmos, loss of visual acuity | Cerebral cryptococcosis,  | Co-trimoxazole, Cephalosporin | Fluconazole | 21.9 | NA |
| 6    | 44; M; MOZ                  | NA             | Positive     | 8,290                     | Fever, headache, behavioral changes | Hypertensive encephalopathy, acute renal failure | None | No | 4.4 | No |
| 7    | 43; M; MOZ                  | NA             | Positive     | 21,100                    | Seizure, headache | Encephalitis, myelitis and encephalomyelitis, unspecified HIV disease | Cephalosporin | No | 0.4 | Yes |
| 8    | 25; M; MOZ                  | NA             | Positive     | >1,000**                  | Fever, dyspnea, diarrhea, headache, abdominal pain, melena | Severe and complicated Plasmodium falciparum malaria, gastroenteritis and colitis of infectious and unspecified origin | Cephalosporin, quinolone, metronidazole, albendazole | No | 0.3 | NA |
| 9    | 35; F; MOZ                  | No             | Positive     | 18,500                    | Fever, headache, visual hallucinations, incoherent speech | Cerebral cryptococcosis, unspecified HIV disease | Co-trimoxazole, cephalexin | Amphotericin B | 3.2 | No |
| 10   | 21; F; MOZ                  | Yes            | Positive     | >100**                    | Dyspnea | Pneumocystosis, fetal death of unspecified cause, anemia unspecified, unspecified HIV disease, pre-eclampsia | Co-trimoxazole, cephalexin, metronidazole | No | 0.9 | NA |
| 11   | 34; F; MOZ                  | Yes            | Positive     | >10,000**                 | Fever, dyspnea, vomiting, headache | Cryptococcal meningitis, unspecified HIV disease, severe acute respiratory syndrome, unspecified hypoglycaemia | Co-trimoxazole, cephalexin | Amphotericin B | 2.8 | NA |
| 12   | 31; F; MOZ                  | Yes            | Positive     | >10,000**                 | Dyspnea, uterine bleeding | Pulmonary edema, unspecified HIV disease; other complications of labor and delivery | Data not available | No | 0.0 | Yes |

Continued
Mycobacterium tuberculosis, cytomegalovirus, esophageal candidiasis, Toxoplasma gondii, and Histoplasma capsulatum) were detected in five cases. Cryptococcus strain characterization. Eight strains were isolated by culture, seven from the CSF and one from the blood. The molecular characterization and antifungal susceptibility of the cryptococcal strains isolated are shown in Table 3. All *C. neoformans* var. *grubii* corresponded to the VNI molecular type, whereas two different genotypes, VGI and VGIV, were identified in the two *C. gattii* isolates. Sero-mating type Aα was found in all the *C. neoformans* isolates. Bα and Cα strains were found among the *C. gattii* strains.

Minimum inhibitory concentrations (MICs) of a variety of antifungal agents were determined (Table 3). All triazole antifungal agents (fluconazole, voriconazole, itraconazole and posaconazole) had MICs less than or equal to the defined epidemiological cut-off values (ECVs)\(^{26}\). One isolate showed a MIC to flucytosine (16 µg/mL), one dilution higher than the ECV (8 µg/mL). Although all the isolates presented a MIC ≤ 1 µg/mL to amphotericin, the MICs of three *C. neoformans* molecular type VNI (1 µg/mL) and two *C. gattii* (1 µg/mL) were one dilution higher than the defined ECVs (0.5 µg/mL)\(^{27}\).

### Discussion

In a large series of nearly 300 autopsies, we performed a thorough histopathological and microbiological analysis of 17 fatal cryptococcal infections, 12 from Mozambique and five from Brazil. Several studies on cryptococcal infection have been carried out in Brazil\(^{14,28}\), but there are no data from Mozambique. Indeed, despite being a highly endemic area for HIV, a literature search was unable to find reports describing the burden of this pathogen in this country. Thus, to our knowledge, this is the largest autopsy-based description of fatal cryptococcal infections in Mozambique. Interestingly, albeit being Maputo and Manaus two sites from different countries in terms of climate and income, some of the results obtained were very similar, such as the HIV prevalence in deceased

| Case | Age (in years), sex and origin | Maternal death | HIV Serology | HIV Viral load (copies/mL) | Main symptoms | Pre-mortem Clinical Diagnoses\(^a\) | Antimicrobial treatment received during illness | Antifungal treatment | Time from admission to death (days) | ART |
|------|-------------------------------|----------------|--------------|--------------------------|---------------|-----------------------------------|-----------------------------------------------|---------------------|---------------------------------|-----|
| 13   | 28; M; BRA                     | NA             | Positive     | 263                      | Dyspnea, diarrhea, vomiting, abdominal pain | Unspecified HIV disease, pulmonary tuberculosis confirmed by sputum microscopy with or without culture, other gastroenteritis and colitis of infectious and unspecified origin, sepsis, unspecified | Quinolones, metronidazole, meropenem | No                  | 5.9                             | Yes |
| 14   | 32; M; BRA                     | NA             | Positive     | NT                       | Vomiting, headache | Unspecified HIV disease, cerebral cryptococcosis | Macrolides, co-trimoxazole | No                  | 12.7                            | No  |
| 15   | 43; M; BRA                     | NA             | Positive     | 1,020                    | Diarrhea, vomiting, headache, asthenia | Unspecified HIV disease, pulmonary tuberculosis, confirmed by unspecifed means, cerebral cryptococcosis, gastrointestinal hemorrhage, unspecified | None | Fluconazole | 4.7 | No |
| 16   | 38; M; BRA                     | NA             | Positive     | 574,000                  | Headache        | Unspecified HIV disease, cerebral cryptococcosis, pneumonia unspecifed, respiratory failure unspecifed | Macrolides, quinolones, co-trimoxazole | No                  | 15.6                            | NA  |
| 17   | 34; M; BRA                     | NA             | Positive     | 17,800                   | Diarrhea, vomiting, abdominal pain | Unspecified HIV disease, pneumonia unspecifed, cerebral cryptococcosis, acidosis | Macrolides | Amphotericin B | 4.1 | No |

Table 1. Clinical characteristics and management of fatal cryptococcal infections. Clinical diagnoses are listed in the order of differential diagnoses included in the clinical charts. Cryptococcus is identified in bold, when considered in the list of diagnoses. \(^a\)As written in the clinical charts by clinicians in charge of patients; M: male; F: female; MOZ: Maputo-Mozambique; BRA: Manaus-Brazil; ART: antiretroviral therapy; NA: not applicable. \(^{**}\)The precise quantification was not possible due to the presence of PCR inhibitors in the plasma, which inhibited the amplification of the internal control of the assay. A minimal viral load was inferred from samples with quantifiable viral loads showing a similar cycle threshold for HIV-1 in the PCR assay; NT: not tested due to insufficient amount of sample.

(Mycobacterium tuberculosis, cytomegalovirus, esophageal candidiasis, Toxoplasma gondii, and Histoplasma capsulatum) were detected in five cases.

**Cryptococcus strain characterization.** Eight strains were isolated by culture, seven from the CSF and one from the blood. The molecular characterization and antifungal susceptibility of the cryptococcal strains isolated are shown in Table 3. All *C. neoformans* var. *grubii* corresponded to the VNI molecular type, whereas two different genotypes, VGI and VGIV, were identified in the two *C. gattii* isolates. Sero-mating type Aα was found in all the *C. neoformans* isolates. Bα and Cα strains were found among the *C. gattii* strains.

Minimum inhibitory concentrations (MICs) of a variety of antifungal agents were determined (Table 3). All triazole antifungal agents (fluconazole, voriconazole, itraconazole and posaconazole) had MICs less than or equal to the defined epidemiological cut-off values (ECVs)\(^{26}\). One isolate showed a MIC to flucytosine (16 µg/mL), one dilution higher than the ECV (8 µg/mL). Although all the isolates presented a MIC ≤ 1 µg/mL to amphotericin, the MICs of three *C. neoformans* molecular type VNI (1 µg/mL) and two *C. gattii* (1 µg/mL) were one dilution higher than the defined ECVs (0.5 µg/mL)\(^{27}\).
| Case | Cause of death (cryptococcal species) | Other diagnosis/ findings | Plasma (PCR Ct value/ CrAg titer) | CSF (PCR Ct value/ CrAg titer) | CNS (PAS/ PCR Ct value) | Lung Right (PAS/PCR Ct value) | Lung Left (PAS/PCR Ct value) | Bone marrow (PAS/PCR Ct value) | Liver (PAS/PCR Ct value) | Spleen (PAS/PCR Ct value) | Uterus (PAS/PCR Ct value) | Kidney ** (PAS) |
|------|--------------------------------------|---------------------------|----------------------------------|-------------------------------|-------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|---------------------|
| 1    | Cryptococcal meningoencephalitis (C. gattii) | Not relevant               | TND/≥1:2560                      | 34.6/≥1:2560                  | Positive/31.6           | Negative/ TND               | Negative/ TND               | Negative/ TND               | Negative/ TND               | Negative/ TND               | Negative/ TND               | Negative/ TND               |
| 2    | Cryptococcal meningoencephalitis (C. neoformans var. grubii) | Spenic tuberculosis, esophageal candidiasis | TND/≥1:2560                      | 26.2/≥1:2560                  | Positive/35.7           | Negative/ TND               | Negative/32.9               | Negative/ TND               | Negative/32.2               | Negative/32.9               | Negative/36.9/NT          | Negative/36.9/NT          |
| 3    | Cryptococcal disseminated disease (C. neoformans var. grubii) | Focal cerebral hemorrhages | 34.5/≥1:2560                      | 27.0/≥1:2560                  | Positive/31.0           | Positive/27.4               | Positive/26.7               | Positive/27.7               | Positive/30.0               | Positive/29.8               | Positive/ NT               | Positive/ NT               |
| 4    | Cryptococcal disseminated disease (C. gattii) | Past malaria               | TND/≥1:2560                      | 26.3/≥1:2560                  | Positive/31.3           | Positive/30.6               | Negative/ TND               | Negative/ TND               | Negative/ TND               | Negative/ TND               | NA/ Negative               | Negative/ TND               |
| 5    | Cryptococcal disseminated disease (C. neoformans var. grubii) | Not relevant | TND/≥1:2560                      | 32.9/≥1:2560                  | Positive/36.2           | Positive/30.0               | Positive/31.6               | Positive/36.6               | Positive/29.4               | Positive/34.3               | NT/TND Positive           | NA/ Negative               |
| 6    | Cryptococcal disseminated disease (C. gattii) | Ischemic cerebrovascular disease, hypertrophic heart, hepatitis | TND/≥1:2560                      | TND/NT                        | Positive/33.1           | Positive/28.7               | Positive/30.8               | Negative/ TND               | Negative/31.5               | Negative/32.6               | NA/ Negative               | Negative/ TND               |
| 7    | Cryptococcal disseminated disease (C. gattii) | Not relevant | NT                               | 29.0/1:160                    | Positive/34.4           | Positive/33.9               | Negative/31.6               | Negative/37.5               | Negative/31.4               | Positive/30.5               | NA/ Negative               | Negative/ TND               |
| 8    | Cryptococcal disseminated disease (C. neoformans var. grubii) | Not relevant | 30.8/≥1:2560                      | 30.0/≥1:2560                  | Positive/29.1           | Positive/23.4               | Positive/24.3               | Positive/25.7               | Positive/31.8               | Positive/22.4               | NA/ Positive               | Positive/ TND               |
| 9    | Cryptococcal meningoencephalitis (C. neoformans var. grubii) | Not relevant | TND/≥1:2560                      | 23.3/≥1:2560                  | Positive/33.5           | Positive/29.9               | Positive/31.0               | Positive/36.1               | Negative/29.7               | Positive/29.3               | NA/ Negative               | Negative/ TND               |
| 10   | Cryptococcal disseminated disease (C. neoformans var. grubii) | Not relevant | 30.5/≥1:2560                      | 25.9/≥1:2560                  | Positive/24.1           | Positive/33.2               | Positive/31.0               | Positive/29.9               | Positive/27.6               | Positive/27.4               | Positive/28.2/NT           | Positive/28.2/NT           |
| 11   | Cryptococcal disseminated disease (C. neoformans var. grubii) | Not relevant | 30.3/≥1:2560                      | 31.1/≥1:2560                  | Positive/30.8           | Positive/27.1               | Positive/27.6               | Positive/34.8               | Positive/30.0               | Positive/29.2               | NT/28.5 Positive           | Positive/28.5/NT           |
| 12   | Cryptococcal disseminated disease (C. neoformans var. grubii) | Esophageal candidiasis    | 35.1/≥1:2560                      | TND/1:40                      | Positive/31.8           | Positive/29.7               | Positive/29.0               | Positive/31.0               | Positive/28.0               | Negative/29.5               | Negative/29.5/NT           | Positive/28.0/NT           |
| 13   | Cryptococcal disseminated disease (C. gattii) | Tuberculosis             | 29.9/1:320                       | NT                            | Positive/34.6           | Positive/28.6               | Positive/24.7               | Negative/ TND               | Negative/ TND               | Negative/31.1               | NA/ Negative               | Negative/ TND               |
| 14   | Cryptococcal disseminated disease (C. neoformans var. grubii) | Not relevant | NT/≥1:2560                       | NT                            | Positive/22.1           | Positive/33.7               | Positive/26.6               | Negative/33.2               | Negative/32.2               | Positive/28.9               | NA/ Positive               | Positive/ TND               |
| 15   | Cryptococcal disseminated disease (C. neoformans var. grubii) | Sepsis due to Streptococcus pneumonia | 32.0/≥1:2560                      | 29.2/≥1:2560                  | Positive/31.6           | Positive/28.8               | Positive/27.6               | Positive/28.9               | Positive/26.4               | NA/ Positive               | NA/ Positive               | Negative/ TND               |
| 16   | Cryptococcal meningitis (Cryptococcus spp.) | Pneumonia (CMV)          | TND/1:1280                       | TND/NT                        | Positive/31.5           | Negative/ TND               | Negative/30.9               | Negative/ TND               | Negative/ TND               | Negative/ TND               | NA/ Negative               | Negative/ TND               |
| 17   | Cryptococcal meningitis (Cryptococcus spp.) | Pulmonary histoplasmosis, Toxoplasmosis | TND/≥1:2560                      | 31.5/1:1280                   | Positive/29.7           | Negative/ TND               | Negative/ TND               | Negative/ TND               | Negative/ TND               | Negative/ TND               | NA/ Negative               | Negative/ TND               |

**Table 2.** Causes of death, histopathological and microbiological findings. CNS: Central Nervous system; CSF: cerebrospinal fluid; CoD: cause of death; TND: Target Not Detected; NT: not tested due to insufficient amount of sample; NA: Not applicable; PAS: Periodic acid–Schiff staining; *PAS positive in placental tissue; PCR: Cycle threshold of a specific real time PCR for Cryptococcus is indicated; **no kidney samples for PCR testing were collected.
patients (over 60%) and the *Cryptococcus* associated mortality in HIV positive patients. In this study *Cryptococcus* was responsible for 10% of the deaths among HIV-infected patients. This figure is in agreement with current estimates of 15% of AIDS-related deaths due to CM. Although treatment with ART has led to an important reduction in the mortality of HIV-infected patients in sub-Saharan Africa, at present, cryptococcal-related death seems to remain similar to 2008 estimates. In agreement with other autopsy series, HIV-associated cryptococcosis is frequently presented as a disseminated infection. **Figure 1.** Cryptococcal disseminated infections: (A) *Cryptococcus neoformans* infecting the central nervous system (hematoxylin and eosin, 400×); (A’) *Cryptococcus gattii* infecting the central nervous system (hematoxylin and eosin, 400×); abundant capsulated yeasts growing extensively in the perivascular spaces, which become cystically dilated; virtually no inflammatory reaction is seen. (B) *Cryptococcus neoformans* infecting the lung (hematoxylin and eosin, 100×); (B’) *Cryptococcus gattii* infecting the lung (hematoxylin and eosin, 100×); abundant capsulated yeasts growing extensively in the alveolar spaces; scant inflammatory reaction is seen. (C) *Cryptococcus neoformans* infecting the placenta (hematoxylin and eosin, 400×); (C’) *Cryptococcus neoformans* infecting the kidney (Periodic Acid Schiff (PAS) Stain, 100×).

| Study number | Sample | Species | Molecular type | Sero-Mating type | Minimum inhibitory concentration (µg/mL) | Amphotericin B | Fluconazole | Voriconazole | Itraconazole | Posaconazole | Flucytosine |
|--------------|--------|---------|----------------|-----------------|-----------------------------------------|----------------|-------------|-------------|--------------|--------------|-------------|
| 1            | CSF    | *Cryptococcus neoformans var. grubii* | VNI            | Aα              | 1 8 0.12 0.12 0.25 | 8              |             |             |              |              |             |
| 2            | CSF    | *Cryptococcus neoformans var. grubii* | VNI            | Aα              | 1 4 0.06 0.03 0.06 | 4              |             |             |              |              |             |
| 3            | CSF    | *Cryptococcus neoformans var. grubii* | VNI            | Aα              | 1 4 0.12 0.03 0.06 | 1              |             |             |              |              |             |
| 4            | Blood  | *Cryptococcus gattii* | VGIV           | Cα              | 1 16 0.25 0.06 0.06 | 4              |             |             |              |              |             |
| 5            | CSF    | *Cryptococcus neoformans var. grubii* | VNI            | Aα              | 1 4 0.06 0.06 0.06 | 4              |             |             |              |              |             |
| 6            | CSF    | *Cryptococcus neoformans var. grubii* | VNI            | Aα              | 1 4 0.06 0.06 0.06 | 4              |             |             |              |              |             |
| 7            | CSF    | *Cryptococcus neoformans var. grubii* | VNI            | Aα              | 1 8 0.06 0.03 0.12 | 16             |             |             |              |              |             |
| 8            | CSF    | *Cryptococcus neoformans var. grubii* | VNI            | Aα              | 1 8 0.06 0.03 0.12 | 4              |             |             |              |              |             |
| 9            | CSF    | *Cryptococcus neoformans var. grubii* | VNI            | Aα              | 0.5 8 0.06 0.03 0.12 | 16             |             |             |              |              |             |
| 10           | CSF    | *Cryptococcus neoformans var. grubii* | VNI            | Aα              | 1 8 0.06 0.03 0.12 | 4              |             |             |              |              |             |
| 11           | CSF    | *Cryptococcus neoformans var. grubii* | VNI            | Aα              | 0.5 8 0.06 0.03 0.12 | 4              |             |             |              |              |             |

**Table 3.** Molecular characterization and antifungal susceptibility of isolated cryptococcal strains. CSF: cerebrospinal fluid.
infections are detected in HIV-infected patients receiving ART\textsuperscript{29}. In our series, only a quarter of the HIV-positive cases (4/16) were under ART. Unfortunately, the duration of ART was only available in one case. This case had a 6-year history of ART and a high viral load, and therefore, likely represents a treatment failure or defaulted. Other explanations for cryptococcal infection in patients receiving adequate ART include recent treatment initiation in patients with very low CD4 counts (late HIV diagnosis) and cases of immune reconstitution inflammatory syndrome\textsuperscript{40}, which might also have been present in our series. Despite progress in ART deployment in sub-Saharan Africa, close to 50% of HIV-infected patients continue presenting to health facilities with advanced HIV disease\textsuperscript{31}, thereby being at high risk of cryptococcal infection and death. In this regard, the CrAg can be detected up to three weeks before the onset of CM symptoms\textsuperscript{32} and therefore, screening of asymptomatic HIV patients followed by preemptive antifungal treatment might identify patients at risk of developing the disease and contribute to reducing CM-related mortality\textsuperscript{33,34}. In the present study, most cases died within the first week of hospital admission, which is in contrast with other series reporting a mean of two weeks between admission and death\textsuperscript{35}. Several factors might have contributed to the rapid fatal outcome in our series. Firstly, the low frequency of clinical suspicion of cryptococcosis may explain the absence of prescribed antifungal treatment in more than half of the patients. On the other hand, some patients may have presented to the hospital with advanced severe stages of cryptococcal infection, considering that seven died within three days of admission (four within 24 hours). The longest hospital admission involved a child who died of \textit{C. gattii} meningitis about two months after hospital admission. \textit{C. gattii} infections are rare in children, and as in the child in this series, the infection often occurs in previously healthy children and presents with CNS involvement\textsuperscript{36}. The little knowledge available about \textit{C. gattii} in sub-Saharan Africa has been obtained from studies performed in South Africa. Interestingly, in our study four out of the 12 cryptococcal infections (25\%) from Mozambique were due to \textit{C. gattii} infections. Although the numbers are small and should be interpreted with caution, these data contrast with previous findings from South Africa, where \textit{C. gattii} represented only 2.4\% of the cryptococcal strains isolated over a two-year period\textsuperscript{36}. \textit{C. gattii} infections have mainly been reported in immunocompetent hosts, until studies from South Africa revealed that it affects immunocompromised patients as well\textsuperscript{37,38}. It has been reported that the VGI \textit{C. gattii} molecular type is much more likely to affect immunocompetent patients than VGI\textsuperscript{39}. However, both genotypes were detected in HIV-infected patients in our study. All strains of \textit{C. neoformans var. grubii} were molecular type VNI and possessed the sero-mating type \textit{Aα}, similar to most of the strains described in South Africa. To our knowledge, this is the first report of \textit{C. gattii} in Mozambique, and the \textit{Bo} and \textit{Co} types detected have been previously reported in South Africa\textsuperscript{39,40}. Although no clinical breakpoints are available for \textit{Cryptococcus}, antifungal susceptibility testing did not suggest clear resistance patterns, with only MICs one dilution above the ECV being found.

A few isolated autopsy reports of fatal \textit{C. gattii} infections in immunocompetent patients have been reported\textsuperscript{41}, which may be explained by the fact that the distinction between \textit{C. gattii} and \textit{C. neoformans} was not performed in many studies. Although the numbers are small, dissemination of the infection seems to be less intense in HIV-infected patients with \textit{C. gattii} compared to \textit{C. neoformans}. Larger studies are needed to better assess the histopathological features of \textit{C. gattii} in HIV-infected patients. Moreover, studies based on post-mortem examinations should be performed to better assess the mortality attributable to specific pathogens in low- and middle-income countries. Accurate mortality data can then impact public health policies, for example, guiding prophylactic and treatment schemes for infectious diseases.

In conclusion, our study highlights the substantial mortality associated with cryptococcal infections among HIV-infected patients, supporting current recommendations\textsuperscript{22} of CrAg screening and preemptive therapy, which is even more relevant in settings in which insufficient pre-mortem clinical suspicion is given to this highly prevalent and life-threatening opportunistic infection.

**Ethics approval and consent to participate.** The study protocol received approval of the National Mozambican Ethics Committee (ref.342/CNBS/13) and the Ethics Committee of the Hospital Clinic of Barcelona (Spain; approved, File 2013/8677). MIA and CDA procedures were only conducted after verbal informed consent was provided by the relatives.

**Data Availability**

All relevant data are within the paper. Any additional data use and transfer is monitored by ISGlobal’s Data Management and Biostatistics Unit (contact e-mail: ubioesdm@isglobal.org).

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Author Contributions
J.C.H., P.C., Q.B., E.L., C.M., J.O. and M.J.M. wrote the main manuscript text. C.C., F.F., D.J., L.L., M.R.I., C.L., A.C. and I.M. provided the data from Mozambique. A.M.P., M.L., W.M. and L.F. provided the data from Brazil. J.C.H., P.C., M.N., I.C., L.Q., F.M., L.M., S.J., A.S., E.L., Q.B., C.M., J.O. and M.J.M. provided the data from Spain. C.C., L.F., A.S., I.M., J.V., Q.B., C.M., J.O. and M.J.M. supervised research activity. All authors reviewed the manuscript.

Additional Information
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