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Elvis Temfack

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Epidemiology and optimal management of cryptococcal meningo-encephalitis associated with AIDS in Cameroon

By Elvis TEMFACK

Doctoral thesis in microbiology and infectious diseases

Directed by Pr Olivier LORTHOLARY

Presented and defended publicly on the 20th of October 2017

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Dedication

To my children: Janelle and Jason.

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List of abbreviations

5FC	Flucytosine
AIDS	Acquired Immune Deficiency Syndrome
AmB	Amphotericin B
ANRS	French National Agency for HIV/AIDS and Hepatitis Research
ART	Antiretroviral therapy
BBB	Blood brain barrier
CM	Cryptococcal meningitis
CNS	Central nervous system
CPC	Centre Pasteur of Cameroon
CrAg	Cryptococcal antigen
CROI	Conference on retroviruses and opportunistic infections
CSF	Cerebrospinal fluid
DC	Dendritic cells
DTA	Diagnostic test accuracy
ELISA	Enzyme linked immunosorbent assay
GalXM	Galactoxylomannan
GM-CSF	Granulocyte monocyte colony stimulating factor
GXM	Glucuronoxylomannan
HIC	High income country
HIV	Human Immune deficiency associated Virus
IAS	International AIDS Society
ICCC	International Conference on Cryptococcus and Cryptococcosis
ICP	Intracranial pressure
IFN	Interferon
IL	Interleukin
IRIS	Immune reconstitution inflammatory syndrome
LFA	Lateral flow assay
LMIC	Low and middle-income countries
LSHTM	London School of Hygiene and Tropical Medicine
MCP	Monocyte chemotactic protein
MIP	Monocyte inflammatory protein

MLST	Multi-locus sequence typing
MP	Mannoproteins
MRC	Medical Research Council
PC	Principal component
PCR	Polymerase chain reaction
POC	Point of care
SRMA	Systematic review and meta-analysis
SSA	Sub Saharan Africa
TNF	Tumour necrosis factor
WHO	World Health Organisation

1. Introduction

1.1. Cryptococcus

1.1.1. Historical background

Cryptococcus spp., a microscopic unicellular fungus, pathogenic to humans, was first identified in peach juice in Italy in 1894 [1]. The first ever described clinical isolate was in the same year, from a sarcoma-like lesion on the tibia of a 31 year old lady [2]. Since discovery, there has been much disarray on its nomenclature, with numerous suggestions, most of which relied on clinical disease features and biochemical characteristics [3]. Initial clinical case descriptions considered it part of the *Saccharomyces* genus but inability to ferment sugars and form ascospores resulted in the name *Cryptococcus neoformans* (*C. neoformans*) in 1901[4]. Since then, there has been incessant growth in interest in this organism and its characteristic tropism for the central nervous system (CNS) first recognised during the period spanning 1915 and 1916 [5, 6]. Its natural environmental habitat was discovered in 1951 in Virginia [7], more than half a century after its discovery, through isolation from soil containing pigeon droppings. Within the same period, serological studies confirmed its antigenic heterogeneity with the discovery of three serotypes: A, B and C in 1950 [8] and a fourth type D in 1968 [9], almost two decades after. At that time, clinical diseases due to *Cryptococcus* spp. were rare until the 70s when immunosuppressive therapy became common and the 80s when it gained unprecedented interest as a cause of morbidity with the discovery of acquired immune deficiency syndrome (AIDS) secondary to human immune deficiency associated virus (HIV) [3].

1.1.2. Updated taxonomy

Cryptococcus spp., for several decades were grouped into two main varieties of the same species: *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* distinguished by their antigenic heterogeneity of which there are five existing serotypes. For var. *neoformans* serotypes A, D and AD (hybrids of A and D) and serotypes B and C for variety *gattii* [10]. Advances in polymerase chain reactions (PCR) led to gene sequence studies of the orotate phosphoribosyl transferase gene (URA5) which found differences between serotypes A and D resulted reclassification of serotype A to a distinct variety: *C. neoformans* var. *grubii*, such that *C. neoformans* var. *neoformans* nomenclature was reserved for serotype D only [11].

Discovery of distinct teleomorphs for varieties *neoformans* and *gattii*, resulted in *C. neoformans* var. *gattii* to be elevated to a species *C. gattii* such that the nomenclature *C. neoformans* as a species was only for serotypes A (var. *grubii*) and D (var. *neoformans*) [12]. More so, molecular clock studies showed that the two species *C. neoformans* and *C. gattii* diverged from each other about 50 million years ago [13], thus more evidence iterating their differences.

With advances in molecular techniques, further characterisation of species described four genotypes each: *C. neoformans*: VNI, VNII, VNIII and VNIV and for *C. gattii*: VGI, VGII, VGIII, and VGIV [14]. Recently, considering data from multi-locus sequence typing (MLST) techniques of cryptococcal isolates, it was suggested that *Cryptococcus* spp. be divided into seven species [15]. Though such a division eases classification, it adds no clinical value as disease expression is not distinguishable. As such, while awaiting to generate more evidence to support the seven-species nomenclature, for practical reasons and ease of communication with clinicians, *Cryptococcus* spp. updated taxonomy recognises two groups: *Cryptococcus neoformans* species complex and *Cryptococcus gattii* species complex [16], to which we will refer to from now on in this write up simply as *C. neoformans* and *C. gattii*.

1.1.3. Ecological niches

As a ubiquitous environmental fungus, *Cryptococcus* spp. has a worldwide distribution especially *C. neoformans* though *C. gattii* for a long time has been considered a tropical and subtropical fungus [3, 17]. With increased surveillance, environmental isolates from Canada, Northern United States of America (USA) and Northern Europe has shown a wider ecological distribution than previously thought [17]. Furthermore, both species have been isolated from decaying plant materials and soil but *C. gattii* has been suggested to favour trees with very waxy cuticles [18, 19].

C. neoformans has been described to be more associated with avian excreta [20, 21] especially pigeons, which may explain why cryptococcosis is more common in densely populated urban areas [17]. So far, there has not been evidence of cryptococcal disease in pigeons and this may be because their body temperatures of 41 - 42°C is higher than that required for yeast growth [3] but isolating *Cryptococcus* spp. from the beaks and feet of these birds suggest that the food they eat from the environment are probably already contaminated by the fungus [22].

1.1.4. Immune response to cryptococcal infection

Initial acquisition of *Cryptococcus* spp. in childhood [23] is from environmental inhalation of desiccated airborne yeast cells into the lungs. These inhaled aerosols are small enough to reach

distal airways where primary immune interaction is with alveolar macrophages or dendritic cells (DC). Studies done in vitro show that DC are involved in detecting, binding, phagocytosis, antigen presentation and activation of T-cells [24, 25]. DC interaction with *C. neoformans* induces cytokine production, of which interleukin (IL)-12 and 23, have been associated with protection against cryptococcosis [26]. Moreover, experienced DC from infected lungs presented with cryptococcal mannoproteins (MP) are capable of ex-vivo activation of MP-specific T-cell [24]. Initial contact of *C. neoformans* with macrophages also results in phagocytosis [27]. However, the fate of this interaction is dependent on the post-activation macrophage phenotype: classical activation results in protection [28] whereas alternate activation results in disease progression [29, 30]. Following interaction with pulmonary innate immune system, adaptive immunity results in the production of antibodies against cryptococcal proteins [23]. From then, the yeast lays quiescent in granulomas in pulmonary lymph nodes awaiting immune depression [31].

Secondary to dissemination from primary pulmonary focus, disease outcome is dependent on the phenotype of systemic response and pro-inflammatory response have been shown to be associated with favourable outcome [32]. CSF cytokine and chemokine profiling in HIV-patients with CM show immune response to be governed by two main principal components (PC): PC1 driven by increased levels of IL-6, interferon (IFN)- γ , IL-8, IL-10, IL-17 and tumour necrosis factor (TNF) and PC2 driven by monocyte chemotactic protein (MCP)-1, monocyte inflammatory protein (MIP)-1- α and granulocyte-monocyte colony stimulation factor (GM-CSF). PC1 pro-inflammatory response correlated with reduced fungal count, high peripheral CD₄ count and CSF white cell count, markers of macrophage activation and better survival [33]. These CSF findings show consistency with systemic responses and correlated with existing evidence of favourable outcome [32].

1.1.5. Virulence factors

Cryptococcus spp., to become pathogenic to humans, developed adaptive survival mechanisms within the hostile environments of immune reaction. In most human fungal infections, initial contact results in pro-inflammatory immune response which is not the case with *Cryptococcus* spp. [34] which has traits capable of immunological masking.

First, its complex polysaccharide capsule rich in glucuronoxylomannan (GXM) and galactoxylomannan (GalXM) extensively shed during infection, inhibit nuclear factors for production pathways of TNF, a pro-inflammatory cytokine [35]. Chitin, a component of the cell wall, is capable of inhibiting DC production of IL-12 and IL-23 which are protective

cytokines [36].

Cryptococcus spp. is capable of intracellular replication in pulmonary macrophages [37]. This ability to escape phagocytic killing through capsule synthesis, melanin formation, urease activity, all combined are protective from reactive oxygen and pH [38, 39]. More so, *Cryptococcus* is capable of perturbing phagosome maturation [40]. Following intracellular replication, it is capable of non-lytic expulsion, known as “vomocytosis” that leaves both macrophage and yeast intact and capable of replication and growth [41].

1.2. Cryptococcosis

1.2.1. Pathogenesis

1.2.1.1. Primary infection

Initial acquisition of *Cryptococcus* spp. is mostly at preschool age through environmental inhalation into the lungs where it causes non-specific unrecognised pneumonia [23]. Within the lungs, interaction with the immune system can lead to the fungus being cleared, disseminated by the bloodstream with monocytes [42] or contained in pulmonary granulomas where it remains quiescent as an asymptomatic latent infection awaiting immune depression for reactivation and dissemination [17, 42].

1.2.1.2. Secondary dissemination from primary focus

A particularity in the pathogenesis of cryptococcal infection is its ability to escape from the primary pulmonary focus into the bloodstream at onset immune depression and disseminate to all organ systems. Following dissemination, though any organ can be affected, *Cryptococcus* spp. is particularly neurotropic leading to its most severe form: cryptococcal meningitis (CM). CM occurs when hematogenous *Cryptococcus* spp. enters the CNS after crossing the impervious blood brain barrier (BBB). Entry of *Cryptococcus* spp. into the CNS is poorly understood and several mechanisms suggested among which proteases mediated paracytosis through inter-endothelial tight junctions [43], hyaluronic acid mediated transcytosis through luminal endothelial cell engulfment [44] and phagocytes mediated “trojan-horse” whereby monocytes containing the fungus cross the BBB [45].

1.2.2. Epidemiology

1.2.2.1. Risk of cryptococcosis

The main risk factor for cryptococcosis is primary or secondary impaired T-cell immune response. Prior to the 1980s, most cryptococcal disease were iatrogenic, secondary to the

extensive use of immunosuppressive therapy [3]. In the 1980s, HIV as a major cause of T-cell immune depression became of major risk factor notably of CM.

In high income country (HIC) settings, those at risk of cryptococcosis are solid organ transplant patients on immunosuppressive therapy, patients with hematologic malignancies, connective tissue diseases, chronic diseases (diabetes, pulmonary or liver diseases) [46-49]. However, in the 1990s, with the spread of the HIV pandemic, cryptococcosis became an AIDS defining illness in this setting [50] whose incidence decreases remarkable with the introduction and scale up of antiretroviral therapy (ART).

In LMIC country settings where HIV infection is the major cause of immune depression, over 90% of CM is HIV related [51]. In these settings, in spite efforts to scale up ART coverage, about 19 – 26% of patients still present to HIV-care with < 100 CD₄ cell/ml [52], a major risk factor for HIV-associated CM. Moreover, in settings with high ART coverage, risk of cryptococcosis remains high among patient failing ART [53]. During cryptococcal disease, components of the polysaccharide capsule known as cryptococcal antigen (CrAg) are shed into biological fluids and detectable weeks prior to onset of symptoms [54]. Detection of CrAg in severely immune depressed HIV patients is associated with 25% risk of CM within the first year of ART [55, 56] in CrAg positive patients. Therefore, CrAg positive HIV patients with <100 CD₄ cell/ml at higher risk of CM with this group presently estimated to represent about 6% of severely immune depressed patients [52].

1.2.2.2. Current burden of HIV-associated cryptococcal meningitis

In 2008, it was estimated that global incidence of HIV-associated CM was 957 900 cases/year resulting in about 500 000 deaths [57]. This estimate which was mostly from cohorts of the pre-ART era, (only three of which from Africa) showed wide confidence intervals due to high levels of uncertainty, has recently been updated taking into consideration decreases in AIDS-related deaths due to the expansion and access to ART [52].

Therefore, 2014 estimates show global annual incidence of 223 000 cases in adults, of which 73% in occurred Sub Saharan Africa (SSA) [52] representing therefore the first cause adult meningitis in Africa. Contrary to what was observed in high income settings, incident CM remains high in LMIC settings with high ART coverage due to patients failing ART [53].

Global annual CM-related mortality was 180 000 of which 75% in Africa. Consequently, CM alone is responsible for about 15% of HIV-related all-cause mortality [52], only second to tuberculosis [58].

1.2.3. Clinical presentation

Clinically, cryptococcosis presents as a more disseminated disease in HIV positive patients in which CM is the most common [59].

1.2.3.1. Pulmonary cryptococcosis

In HIV-associated CM, pulmonary involvement occurs in 10 to 55% of patients, with a clinical spectrum ranging from asymptomatic to acute respiratory distress syndrome and may be misdiagnosed as pulmonary tuberculosis [60]. However, the incidence of cryptococcal pneumonia in the absence of meningitis remains unknown in high burden areas due to lack of diagnostic facilities. A South African post-mortem study of lung tissues of miners showed high incidence of pulmonary cryptococcosis and high rates of coinfection with other infections, warranting the consideration of dual pathology in AIDS patients when diagnosing infections such as tuberculosis [61].

During the Vancouver outbreak of *C. gattii*, pulmonary involvement was the most common site of disease in 87% of patients and in Australia, isolated pulmonary as well as pulmonary and associated CNS diseases were reported in 12% and 51% of cases respectively [62].

Nevertheless, pulmonary cryptococcosis has no pathognomonic features distinguishable from other pneumonia and large pulmonary cryptococcomas may be misdiagnosed as tumor.

1.2.3.2. Cutaneous

Though rare, primary cutaneous cryptococcosis can occur following direct inoculation by infected objects resulting to localised lesions at the inoculation point. However, generalised cutaneous cryptococcosis should be presumed to be secondary to disseminated disease. In disseminated disease, the skin is among the most common site after the CNS and may present with molluscum contagiosum-like lesions in immune depressed patients [63]

1.2.3.3. Disseminated disease (fungemia)

Fungemia most commonly precedes CNS involvement and patient with positive blood culture should be explored for CNS and considered of combination antifungal therapy. Disseminated disease is frequent in HIV patients especially of male sex [59].

1.2.3.4. Cryptococcal meningitis

Clinically, HIV-related CM onset is insidious, could be asymptomatic or present with non-specific signs and symptoms among which fever, nausea and vomiting. Most commonly, neurological symptoms includes headache [64] and altered mental status [65]. With associated raised intracranial pressure (ICP), common in 60 – 80% of cases, especially in patients in sub-

Saharan Africa (SSA) [66], most patients may present visual impairment due to involvement of optic nerves and tracts [67].

1.2.4. Diagnosis of cryptococcosis

1.2.4.1. Microscopy

Diagnosis of CM by India Ink staining of CSF is by identification of rounded cells measuring about 4 – 6 μm in diameter, around which a clear halo representing the thick capsule can be observed. Sensitivity of this technique is around 86% [68, 69] but could be much lower in HIV-negative patients due to low fungal burden [59]. As such, sensitivity of microscopic examination may be increased by staining CSF sediments after centrifugation of at least 1ml of CSF.

1.2.4.2. Culture

Definitive standard diagnosis of cryptococcosis reposes on cultures of biological samples (CSF, urine, blood, sputum or fluid of bronchoalveolar lavage) in Sabouraud dextrose agar [70]. *Cryptococcus* spp. also grows when incubated at 30°C rather than 37°C on usual media used for bacteria and fungi except in those with cycloheximide. Culture might be negative in case of exposure to antifungal therapy or may need longer incubation periods up to 3 weeks.

1.2.4.3. Antigen detection

CrAg is a complex polysaccharide of cryptococcal capsule which is shed in biological milieus and can be detected as markers of fungal presence. Detection of CrAg relies on specific antibody-antigen using different principles among which latex agglutination (LA) enzyme-linked immunosorbent assays (ELISA) and most recently lateral flow assay (LFA). Though these tests can be performed on serum and/or CSF, most ELISA tests are strictly required to be performed only on serum; LA and LFA in plasma, serum and urine. However, LFA has been shown to show many false positive results in urine resulting in urine not to be a predilected specimen for CrAg testing with LFA [71]. CrAg detection in serum is presumptive of cryptococcosis especially in HIV patients and detection in CSF is diagnostic of CM [70, 72]

1.2.5. Treatments of cryptococcosis

1.2.5.1. Prophylaxis

Prevention of cryptococcosis so far has mostly been for HIV patients. Primary oral fluconazole-based prophylaxis was suggested to all patients presenting with severe immune depression but this strategy was not sustainable, exposed patients to chemotherapy without proof of disease,

had no effect on mortality and so was not widely practiced even though there were some benefits in decreasing incident CM incidence [73].

Presently, preventive strategies rely on targeted fluconazole-based pre-emptive treatment of asymptomatic CrAg positive patients following pre-ART systematic CrAg screening in those presenting with < 100 CD₄ cells/ml. Fluconazole in this case is offered at a dose of 800mg/day for two weeks then 400mg/day till ten weeks and 200mg/day till immune reconstitution [72]. However, this dose remains provisional with little evidence on its effectiveness because so far there has not been any trial to compare pre-emptive therapy to no pre-emptive therapy in CrAg positive patients.

1.2.5.2. Curative

Treatment of CM relies on a limited arsenal of medications, belonging to three main classes: polyenes (amphotericin B [AmB]), azoles (fluconazole) and pyrimidine analogue (flucytosine [5FC]), all of which are many decades old [74].

Present World Health Organisation (WHO) guidelines [72], recommend treatment of CM in three phases: induction, consolidation and maintenance phases. Standard induction treatment relies on a combination of intravenous infusions of AmB at 1mg/kg/day and oral flucytosine at 100 mg/kg/day, administered for 14 days. Consolidation therapy relies on fluconazole alone at 400 – 800 mg/day till 10 weeks followed by maintenance therapy of 200 mg/day [72] till immune reconstitution (CD₄ count >200). However, with respect to induction treatment, these guidelines provide conditional recommendations depending on the availability of AmB and/or 5FC and the ability of settings to monitor and manage AmB related toxicities. In routine care in African settings, where monitoring of AmB related toxicity is not common practice, as many as 16% and 32% develop AmB-related anaemia and kidney impairment, respectively [75].

Immediate ART initiation following an episode of CM per these guidelines is not recommended due to high risk of life threatening immune reconstitution inflammatory syndrome (IRIS) and should be deferred by 5 weeks following induction treatment [76].

1.2.5.3. Adjunctive treatment of raised intracranial pressure

Raised ICP with CSF opening pressures greater than 20 cm H₂O is common in CM and has been shown to be associated with neurological symptoms of visual [67] and hearing impairment [77] and a poor outcome following treatment. As such opening pressure (OP) should be measured at baseline and subtractive LP for CSF drainage done every 24-48h, in case OP is >25 cmH₂O with the aim to getting the OP to <20 cmH₂O or to 50% of initial OP. During each subtractive LP, a maximum of 30ml daily CSF drained seems to be safe. Neurosurgical

shunting could be considered for patients with persistently high OP albeit daily LP for CSF drainage [78].

1.3. Overview on cryptococcosis in Africa

In Africa, where more than two thirds of the global burden of HIV is found [58], over 90% of cryptococcosis is HIV-associated, representing about 75% of worldwide CM burden [51]. Globally, CM is responsible for about 15% of HIV-related all-cause mortality, 73% of which occurs in Africa [52]. This unacceptably high mortality in Africa is driven by many factors among which health system related, patient related and those related to the severity of the disease. As a result, identifying and addressing these factors is crucial in reducing CM related mortality. These factors include

1. Low ART coverage and poor adherence

Ideally, forestalling severe immune depression by timely ART is the best preventive strategy for HIV-related CM as shown in high income settings where massive ART roll out has led to remarkable decrease in the incidence of CM [79]. Recently in 2016, global estimates show about 19.5 million of the 36.7 million people living with HIV were accessing ART, therefore coverage of about 50% [58]. This implies one in two HIV patients at risk of progression to severe immune deficiency, disproportionately higher in Africa due to higher HIV burden. More so, in settings with almost universal ART coverage like Botswana, CM morbidity and mortality remains high especially in patients failing ART [53] thus need optimal adherence and retention to care

2. Low implementation of systematic CrAg screening and pre-emptive therapy

Cryptococcosis being a sub-acute infection, is peculiar in that CrAg can be detected in blood many weeks before the onset of symptoms [54] and serum CrAg positive patients are at 25% higher risk of developing CM within the first year of ART [55, 80, 81]. As such, systematic pre-ART CrAg screening and pre-emptive fluconazole is recommended for those presenting with <100 CD₄ cell counts. Though promising as a strategy to decrease incident CM [82, 83], especially when associated with timely ART initiation, its uptake still lags in many countries.

3. Late presentation to care

Albeit efforts to scale up ART coverage, as many as 25% of patients present to HIV care with severe immune deficiency (<100 CD₄ cells/ml) [52]. In many LMIC, a large proportion of CM cases inaugurate HIV/AIDS diagnosis [84], with patients presenting already with signs disease severity [72, 85] like altered mental status, mostly associated poor outcome [86].

4. Delayed diagnosis

Clinically, the onset of CM is insidious. Symptoms are usually non-specific necessitating a low a low threshold of clinical suspicion for timely diagnosis which could delay diagnosis to about a week even in high income settings [87]. In LMIC settings, delay is longer due to lack of material for LP, low acceptability of LP [82] and unavailability of confirmatory diagnostic material.

5. Unavailability of recommended treatment

In most LMIC with the highest burden of CM [52] recommended AmB plus 5FC for inductive treatment [72] is not available, therefore inductive treatment relies on fluconazole monotherapy, known to be ineffective event at high doses for the treatment of CM [88, 89].

6. Incapability to monitor AmB-treatment related side effects

AmB required for inductive CM treatment is potentially associated with adverse events, some of which could be life threatening, thereby necessitating closed laboratory monitoring and treatment [72]. These side effects could be limiting especially in settings without capacity of laboratory monitoring and treatment.

7. Poor management of CM-related raised intracranial pressure

Raised ICP which occurs frequently in CM, is associated with high mortality [77, 86] requiring closed monitoring of CSF opening pressures during LP, with eventual subtractive CSF drainage when opening pressures are >20 cm of H₂O. However, in many settings with high burdens of CM, manometers and personnel trained for the procedure are not readily available.

1.4. Situational analysis in Cameroon and rationale of the thesis project

In Cameroon, where about a million people are HIV-infected, over 25% of CNS diseases is due to CM [90, 91]. HIV diagnosis and management is based on local guidelines, constantly updated following WHO recommendations. However, there are not local guidelines for the prevention, diagnosis and treatment of HIV-associated CM.

Consequently, clinical suspicion, diagnosis (mostly presumptive based on signs and symptoms) and treatment of CM are entirely at the discretion of the treating physician. At clinical suspicion of CM, confirmatory diagnosis in most hospitals where LP is feasible, relies solely on Indian ink staining of CSF (albeit its relatively low sensitivity [69]) not always routinely available in most hospitals. CrAg tests are not available and systematic fungal culture of CSF is not common practice in most hospitals but could be available on request in many reference laboratories like Centre Pasteur of Cameroon (CPC). AmB and flucytosine recommended for induction treatment are not registered in the country, thus unavailable. Consequently, upon presumptive or confirmatory diagnosis, CM treatment relies solely on low-dose fluconazole

monotherapy, obtained through out-of-pocket payment by patients, resulting in poor management and high in-hospital mortality of over 50% even in reference hospitals [64]. Therefore, in a nutshell, there are no predefined algorithms in the continuum of care from clinical suspicion of CM to the inevitable fatal outcome. This implies that in Cameroon where HIV has been defined as a major public health problem, CM could be a major under-estimated driver of morbidity and mortality. As such, there is need for local prevention, diagnostic and treatment strategies as part of a comprehensive HIV health care package aimed at decreasing all-cause HIV-related mortality.

Therefore, to contribute to the integrated management of HIV and its related opportunistic infections in Cameroon, we proposed to carry out, an international interventional PhD thesis project entitled "*Epidemiology and optimal management of cryptococcal meningo-encephalitis associated with AIDS in Cameroon*".

1.5. Objectives of the thesis

1.5.1. General objective

To optimise the management of HIV-associated cryptococcal meningitis in Cameroon

1.5.2. Specific objectives

- To evaluate systematic cryptococcal antigen screening and pre-emptive fluconazole based therapy as a strategy to reduce morbidity and mortality associated with HIV-related cryptococcal meningitis
- To assess the diagnostic accuracy of currently available cryptococcal antigen detection tests and evaluate the contribution of a new semi-quantitative Biosynex CryptoPS test in the screening and diagnosis of cryptococcosis in resource limited settings.
- To determine the most adapted antifungal combination therapy for the inductive treatment of HIV-associated cryptococcal meningitis in Africa
- To evaluate the tolerability of amphotericin B deoxycholate in patients treated in the phase three trial on initial treatment of cryptococcal meningitis in sub-Saharan Africa
- To determine long term mortality and disability of CM in patients surviving an initial episode of CM

1.6. General overview on the thesis

To attain our objectives, operational studies involving a wide network of international collaboration were carried out. These included:

1. Two prospective interventional studies

- The “Pre-antiretroviral Therapy Cryptococcal Antigen Screening in AIDS” (PreCASA) study [92]
- The “Advancing Cryptococcal meningitis Treatment for Africa” (ACTA) multicentre trial “MRC/ANRS 12275 ACTA” [93] trial

2. Three systematic reviews

- The diagnostic accuracy of cryptococcal antigens detection in HIV associated cryptococcal meningitis: systematic review and meta-analysis (SRMA).
- Routine pre-antiretroviral therapy cryptococcal antigen (CrAg) screening as a strategy to reduce HIV-associated cryptococcal meningitis-related morbidity and mortality: systematic review and meta-analysis.
- Long term mortality and disability in cryptococcal meningitis: a systematic review.

3. One secondary analysis of clinical trial data

- Tolerability of amphotericin B deoxycholate (AmBd) in patients treated for HIV-associated cryptococcal meningitis in the ACTA phase III trial: the AmBiTol Study

4. An advocacy

- Cryptococcal meningitis, a neglected NTD?

2. Methodological approach to HIV-associated cryptococcal meningitis

2.1. Cryptococcal antigen detection and prevention: systematic review

2.1.1. Summary

Background

Routine cryptococcal antigen (CrAg) screening and targeted pre-emptive fluconazole therapy in antiretroviral naive HIV-infected adults with less than 100 CD4 cells/ μ l seems promising to reduce the burden of cryptococcal meningitis (CM).

Methods

We systematically assessed the prevalence of CrAg positivity, the prevalence of asymptomatic CM in CrAg-positives, the incidence of CM during follow-up and all-cause mortality in screened participants. We searched MEDLINE, EMBASE, and Web of Science for studies published from January 1981 to February 2017 in which routine CrAg screening was done. We assessed included studies for risk of bias using the Joanna Briggs Institute checklist for cohort studies. We used random-effects meta-analysis to synthesise results.

Results

We included 20 studies, encompassing 11,163 participants from 13 countries. Risk of bias was low. Prevalence estimates of CrAg positivity and asymptomatic CM in CrAg-positives were 6% (95%CI: 5 – 7) and 36% (95%CI: 12 – 60), respectively. Without pre-emptive fluconazole therapy, the incidence of CM during follow-up was 21.4% (95%CI: 11.6 – 34.4) and 0.4% (95%CI: 0.1 – 1) in CrAg-positives and CrAg-negatives, respectively (risk ratio [RR] 52.7, 95%CI: 6.4 – 431.2; $p=0.0002$). With targeted pre-emptive fluconazole therapy, the incidence of CM during follow-up was 6.6% (95%CI: 3.8 – 10.5) and 0.3% (95%CI: 0.2 – 0.5) in CrAg-positives and CrAg-negatives, respectively (RR 15.6, 95%CI: 4.5 – 53.8; $p=0.0001$). Two studies in which lumbar puncture was offered to CrAg-positives, the incidence of CM during follow-up further dropped to null. However, even with post-screening lumbar puncture, all-cause mortality remained higher in CrAg-positives than in CrAg-negatives (RR 2.3, 95%CI: 1.5 – 3.4; $p<0.001$).

Conclusion

Routine CrAg screening and targeted pre-emptive fluconazole therapy has the power to dramatically reduce the incidence of CM, but has unclear effects on all-cause mortality rates.

Key words: Cryptococcal antigen, screening, pre-emptive fluconazole, meningitis, latex agglutination, lateral flow assay.

2.1.2. Draft article 1: for submission to the Lancet Public Health

Impact of routine cryptococcal antigen screening and targeted pre-emptive fluconazole therapy in antiretroviral naive HIV-infected adults with less than 100 CD4 cells/ μ l: Systematic review and meta-analysis

INTRODUCTION

Cryptococcal meningitis (CM) is due to a ubiquitous environmental encapsulated yeast, *Cryptococcus* spp, and occurs primarily in patients with defective cell-mediated immunity [48, 94]. Consequent to the HIV pandemic, there has been a remarkable surge in the incidence of CM, especially in Sub-Saharan Africa [52, 57]. In such settings, over 90% of CM cases occur in HIV-infected patients [51, 95]. With the introduction of antiretroviral therapy (ART) in the 1990s, the incidence of CM has declined in high-income countries (HIC) [96]. However, in low- and middle-income countries (LMIC), around 20% of patients still present to HIV care units with less than 100 CD₄ cells/ μ l, a major risk factor for developing CM [52, 97]. In LMIC settings, CM is estimated to account for around 15% of HIV-related mortality [52]. In recent Sub-Saharan African cohorts, in-hospital case fatality rates of CM range between 30% and 60% [64, 95, 98-100].

There is therefore an urgent need for effective strategies to reduce the burden of CM [56]. The “blanket” strategy relies on using fluconazole-based primary prophylaxis in all patients with less than 100 CD₄ cells/ μ l [101]. Though this strategy proved able to reduce the incidence of CM [73], it has not been widely implemented because of lack of evidence of survival benefits, concerns regarding pharmacokinetic drug interactions, potential for inducing resistance to fluconazole, teratogenicity, and high cost. This prompted experts to suggest targeted pre-emptive fluconazole therapy, i.e. the early identification of patients at higher risk of CM who are more likely to benefit from treatment.

Cryptococcus contains a capsular polysaccharide, known as cryptococcal antigen (CrAg), which can be detected in blood many weeks to months prior to onset of CM [54]. Evidence

suggests that without fluconazole therapy, CrAg-positive patients have up to 25% increased risk of CM in the first year of ART [55, 56]. Thus, in 2011, the World Health Organisation (WHO) [102] suggested routine CrAg screening in ART-naïve HIV-infected adults with less than 100 CD₄ cells/ μ l, using either latex agglutination (LA) or lateral flow assay (LFA) procedures [102, 103]. Following WHO's advice, CrAg-positive patients should then be offered pre-emptive fluconazole therapy, at a provisional tapering dose of 800 mg/day for two weeks, then 400 mg/day for eight weeks, and then 200 mg/day until they have more than 200 CD₄ cells/ μ l [102]. However, this recommendation remains provisional because the optimal antifungal regimen for this population remains to be determined [104].

CrAg screening with targeted pre-emptive fluconazole therapy seems attractive and cost-effective [105-108], but how best to implement it in overstretched, under-resourced high disease burdened health care settings remains a challenge. Nevertheless, it is incorporated into several national HIV care guidelines, both in LMIC (e.g., Botswana, Kenya, Mozambique, Namibia, Rwanda, South Africa, Swaziland, Uganda) and HIC settings (e.g., USA and France) [52, 109]. A systematic assessment of the impact of this strategy is lacking. We performed a systematic review and meta-analysis to assess four key clinical outcomes of routine cryptococcal antigen screening and targeted pre-emptive fluconazole therapy in ART-naïve HIV-infected adults with less than 100 CD₄ cells/ μ l: the prevalence of CrAg positivity, the prevalence of asymptomatic CM in CrAg-positives, the incidence of CM during follow-up and all-cause mortality in screened participants.

METHODS

Search strategy and study selection

A medical information specialist (RS) developed a comprehensive search strategy to identify published and unpublished studies in MEDLINE, EMBASE, and Web of Science. Medical subject headings (MeSH) and keywords included: “cryptococcal antigen”, “cryptococcal

surface polysaccharides”, “cryptococcal meningitis”, “HIV”, “screening”, “detection”, “latex agglutination”, “lateral flow assay”, as shown in Appendix 1. To avoid missing relevant studies, we did not use methodological filters. Searches were run from January 1981 (year of first HIV case) through February 2017. References of included studies and previous reviews on the subject were screened for eligibility. Reports that cited included studies were also searched on Google Scholar. Conference proceedings of the Conference on Retroviruses and Opportunistic Infections (CROI), the International Conference on Cryptococcus and Cryptococcosis (ICCC), and the International AIDS Society (IAS) conference were screened from 2010 onwards.

Two review authors (ET, JJBR) independently screened studies by title and abstract and assessed full texts of potentially relevant studies. Discrepancies were resolved by discussion. When consensus was not reached, study inclusion was further discussed with a third author (JFC). Study selection was done using Rayyan systematic reviews software (<http://rayyan.qcri.org>).

We included cross-sectional studies, randomised controlled trials (RCT), and cohort studies (retrospective and prospective) in which study participants were offered CrAg screening using LA and LFA procedures. Case-control studies and case reports were excluded. Study participants had to be HIV-infected adults (age >18 years) presenting to HIV-care programs with less than 100 CD₄ cells/μL, naïve to ART, with no symptoms suggestive of CM, and in whom serum CrAg screening was done prior to ART initiation. There was no country restriction. Only studies published in English, French and Spanish were included.

In this review, the main intervention of interest was pre-emptive fluconazole therapy in CrAg-positive patients. However, to our knowledge, there is no RCT evaluating the effectiveness of this intervention. Such a trial would be unethical because there is enough clinical evidence to

suggest that fluconazole therapy may reduce the risk of CM in severely immunosuppressed HIV-infected patients [73]. Consequently, in the present review, the impact of this intervention was evaluated based on observational studies.

Our clinical outcomes of interest were: (i) the prevalence of CrAg positivity in screened participants, (ii) the prevalence of asymptomatic CM (ascertained by positive fungal culture and/or Indian ink staining and/or CrAg in cerebrospinal fluid [CSF]) in CrAg-positive patients, (iii) the incidence of CM during follow-up, and (iv) all-cause mortality during follow-up.

Data extraction and quality assessment

For each study, the following data were extracted onto a pre-specified and piloted data extraction form:

- Study characteristics: first author, publication year, report type (journal article, short communication, conference abstract), study design (RCT, cohort, prospective vs. retrospective, cross-sectional), setting (country, region, level of care, continent), inclusion and exclusion criteria;
- Participant characteristics: number of participants, age (mean or median), sex ratio, proportion of ART-naïve participants, CD4 counts (mean or median), number and proportion of participants with less than 100 CD₄ cells/μL, other relevant characteristics;
- CrAg screening test: method (LA or LFA), commercial name if available;
- CrAg screening outcome: number of screened participants; number and proportion of CrAg-positive results;
- Interventions offered to CrAg-positive patients: lumbar puncture (number of patients eligible, number who accepted, number of asymptomatic CM cases), pre-emptive fluconazole therapy (offered or not, number of patients offered fluconazole, initial dose offered, duration), ART (median time to initiation if available);
- Clinical outcomes within follow-up: incidence of CM (number and proportion in CrAg-

positive and CrAg-negative participants), all-cause mortality (number and proportion in CrAg-positive and CrAg-negative participants), number lost to follow-up within each group, if reported;

- Any other information of relevance (e.g., funding).

We assessed risk of bias only for studies in which pre-emptive fluconazole therapy was offered to CrAg-positive participants. For this purpose, we developed a quality assessment tool based on the Joanna Briggs Institute checklist for cohort studies (appendix 2) [110]. The main components of the review question were: study population (HIV-infected adults with less than 100 CD₄ cells/ μ L), exposure (CrAg status and the method used to determine this status), intervention (targeted pre-emptive fluconazole therapy, ART to screened patients), and the outcomes of interest within follow-up time (incident cases of CM and all-cause mortality). For each study, we assessed patient selection bias, treatment allocation bias, outcome assessment bias and completeness of outcome data bias. Where insufficient information was reported we contacted study authors for clarification.

Statistical analysis

Data were pooled using standard random-effects meta-analysis for proportions to calculate the prevalence of CrAg positivity in screened participants and the prevalence of asymptomatic CM in CrAg-positive participants, together with their 95% confidence interval (95%CI). In studies where participants were followed-up after screening, random-effects models were used to estimate the incidence of CM and all-cause mortality during follow-up and to estimate risk ratios comparing CrAg-positive to CrAg-negative participants; this analysis was stratified according to the type of interventions offered to CrAg-positive participants (i.e., no pre-emptive fluconazole, any pre-emptive fluconazole, pre-emptive fluconazole initiated at <800 mg/day, pre-emptive fluconazole initiated at 800 mg/day, pre-emptive fluconazole initiated at 800 mg/day after post-screening lumbar puncture). Heterogeneity was evaluated graphically

by observing the forest plots and by calculating I^2 statistics. Statistical analysis involved STATA 13.0 (Statacorp, Texas, USA) and Review manager (Revman) version 5.3 [111].

RESULTS

Results of the search

The electronic search run on the 20th of February 2017 identified 1,898 citations (245 duplicates). Based on title and abstract screening, 1,609 citations were excluded (Figure 1). Based on further assessment of 44 full texts, 20 studies were included for estimating the prevalence of CrAg positivity [56, 81-83, 104, 105, 108, 112-124], 5 to evaluate the prevalence of asymptomatic CM in CrAg-positive patients [82, 83, 112, 114, 121], 3 to evaluate the incidence of CM and all-cause mortality in the context of no fluconazole pre-emptive therapy [56, 113, 116], and 10 to evaluate the incidence of CM and all-cause mortality in the context of pre-emptive fluconazole therapy [81-83, 104, 105, 114, 120-123].

Prevalence of CrAg positivity in screened participants

Twenty studies originating from 13 countries were included [56, 81-83, 104, 105, 108, 112-124], among which 14 (70%) were cohorts, 3 (15%) randomised trials, and 3 (15%) cross-sectional studies (Table 1). Among 14,144 participants screened for CrAg, 11,163 had less than 100 CD4 cells/ μ L. CrAg screening was done with LA in 10 (50%) studies, and LFA was used in the rest. Screening was performed at the point of care in 12 (60%) studies and on stored sera in 8 (40%). The prevalence of CrAg positivity ranged from 2 to 21%; the pooled prevalence of CrAg positivity was 6% (95%CI: 5 – 7).

Prevalence of asymptomatic CM in CrAg-positive participants

Following CrAg screening, lumbar puncture was offered to CrAg-positive participants in 5 studies, for a total of 139 asymptomatic CrAg-positive patients [82, 83, 112, 114, 121]. Of these 139 patients eligible for lumbar puncture, 84 (60.4%) consented. The pooled prevalence of asymptomatic CM in CrAg- positive participants was 36% (95%CI: 12 – 60).

Incidence of CM during follow-up

Estimates of the incidence of CM during follow-up are presented in Table 2 and Figure 4. Pooling three cohort studies [56, 113, 116] in which 1,057 participants were screened for CrAg and no fluconazole was offered to CrAg-positives, the pooled incidence of CM during follow-up was 21.4% (95%CI: 11.6 – 34.4) and 0.4% (95%CI: 0.1 – 1) in CrAg positives and CrAg negatives, respectively (RR 52.7, 95%CI: 6.44 – 431.2, p=0.0002).

Pooling nine studies [81, 82, 104, 105, 114, 120-123] with 4,708 participants in which CrAg positive patients received pre-emptive fluconazole at any dose, incidence of CM was 6.6% (95%CI: 3.8 – 10.5) and 0.3% (95%CI: 0.1 – 0.5) in CrAg positive and negative participants, respectively (RR 15.6, 95%CI 4.5 – 53.8, p=0.0001).

On stratified analysis, the incidence of CM in CrAg positive participants ranged from 6% (95%CI: 3.2 – 10.3) in those receiving fluconazole at 800 mg/day [81, 82, 104, 121, 122] to 9.1% (95%CI: 2.5 – 21.7) in those receiving fluconazole at an initial dose less than 800 mg/day [105, 114, 123, 125]. In this analysis, the incidence in CrAg negatives was consistently less than 1%.

In two studies [82, 121] in which 810 participants were screened and lumbar puncture was offered to CrAg positive patients, the incidence of CM was 0% (95%CI: 0 – 0.1) and 0.4% (95%CI: 0 – 1) in CrAg positive and CrAg negative patients, respectively (RR: 5.7, 95%CI: 0.7 – 49.8, p=0.1).

All-cause mortality during follow-up

Estimates of all-cause mortality rates during follow-up are presented in Table 3 and Figure 5. Pooling two [56, 113] of the three studies (one did not report mortality [116]) in which CrAg positive patients did not receive pre-emptive fluconazole following screening, all-cause mortality was 32.6% (95%CI: 19.1 – 48.5) and 8.8% (95%CI: 6.7 – 11.2) in CrAg-positive and CrAg negative patients, respectively (RR: 3.6, 95%CI: 2.2 – 5.8, p<0.0001). Pooling seven

studies [81-83, 104, 105, 120, 121] with 4,140 participants in which CrAg positive patients received pre-emptive fluconazole at any dose, mortality was 17.2% (95%CI: 12.8 – 22.2) and 16.9% (15.8 – 18.2) in CrAg positive and CrAg negative participants, respectively (RR: 1.7, 95%CI: 0.7 – 4.2, p=0.23).

On stratified analysis, mortality in CrAg positive patients ranged from 16.2% (95%CI: 11.8 – 21.5) in those who received pre-emptive fluconazole at initial dose of 800 mg/day [81-83, 104, 121] to 25.9 (11.1 – 46.3) in those receiving at less than 800 mg/day initial dose [105, 120] and there was no significant risk difference in mortality between CrAg positive and negative patients (p=0.42).

In three studies [82, 83, 121] in which 1,538 participants were screened for CrAg and lumbar puncture was offered to CrAg positive patients, all-cause mortality was 25% (95%CI: 15.5 – 36.6) and 11.8% (95%CI: 10.1 – 13.6) in CrAg positive and CrAg negative patients, respectively (RR: 2.3, 95%CI: 1.5 – 3.4, p<0.001).

DISCUSSION

Main findings

This systematic review and meta-analysis shows that (i) the prevalence of CrAg positivity in asymptomatic HIV-infected patients with less than 100 CD₄ cells/ μ L is around 6%, (ii) among CrAg-positives, the prevalence of asymptomatic CM is 36%, (iii) the incidence of CM in CrAg-positives drops from 21.4% without pre-emptive fluconazole therapy, to 6% with pre-emptive fluconazole therapy initiated at 800 mg/day, (iv) the impact of CrAg screening and pre-emptive fluconazole therapy in terms of mortality remains unclear.

Implications for practice

We bring evidence that targeted pre-emptive fluconazole therapy may lead to the reduction of the incidence of CM from 21.4% to 6%, which corresponds to a 70% relative decrease. When CrAg-positive patients were offered post-screening lumbar puncture, the incidence of CM even

reduced to less than 1%, which is comparable to that observed in CrAg-negatives. This therefore implies that clinicians should systematically offer lumbar puncture to CrAg-positives, otherwise some patients would be put on supposed pre-emptive therapy which is sub-optimal inductive antifungal treatment with fluconazole monotherapy, known to be ineffective in CM even at highest dosages [88, 89]. In other words, the observed incident CM cases during follow-up albeit pre-emptive fluconazole therapy might simply be caused by insufficient treatment, together with probable immune reconstitution inflammatory syndrome. We therefore suggest that the objective is not only to identify CrAg-positive patients, but also, among them, those who have asymptomatic CM. Patients with asymptomatic CM should be treated according to current standards of care, while fluconazole pre-emptive therapy should be restricted to those without CSF evidence of CM.

In studies reporting the experience of routine CrAg screening and targeted fluconazole therapy in LMIC settings, we found little statistical heterogeneity, suggesting similarities across these studies in the overall implementation of the screen-and-treat strategy: tests used, classification of patients as CrAg-positives or -negatives, fluconazole to CrAg-positive patients, post-screening ART initiation, follow-up and reporting of ascertained CM cases over time. However, there was much heterogeneity in the way fluconazole was offered to CrAg-positive patients in terms of dosage and duration (Table 1). Few studies provided fluconazole at the WHO-suggested tapering dose and duration [81-83, 122]. In some, fluconazole was initiated at 800 mg/day and provided for four weeks only [121] or for two weeks then 400 mg/day for another two weeks and stopped [104]; these short courses seemed to be due to local realities of insufficient fluconazole availability. This shows that for targeted pre-emptive therapy to be effective as a preventive strategy for CM, readily available and sustainable fluconazole is a prerequisite, especially as CrAg point-of-care tests are becoming more available [103, 126] and accepted by clinicians and patients.

Implications for research

Given that most studies show moderate lumbar puncture feasibility and acceptance (60%), there is an urgent need for more acceptable methods for identifying those with asymptomatic CM among CrAg-positives. With existing evidence of association between serum CrAg titres and asymptomatic CM [80-82], systematic post-screening CrAg quantification can be done, and a threshold defined beyond which patients could be considered for recommended inductive combination antifungal therapy (i.e., amphotericin B plus flucytosine). Available evidence for such a threshold is around 1:160 [80, 82] and a recent Ugandan study [81] showed strong association between this titre level and incident CM within weeks of ART induction. Future research should aim at evaluating whether semi-quantitative point-of-care CrAg tests capable of identifying patients with high titres [127] would increase the likely effectiveness of pre-emptive therapy.

With regards to the effect of pre-emptive fluconazole on all-cause mortality, there was much statistical heterogeneity, probably because of heterogeneous study design and because of differences in settings. Even though post-screening lumbar puncture prior to pre-emptive high dose fluconazole probably reduced CM-specific mortality, nevertheless, CrAg-positive patients still had more than two-fold likelihood of death than CrAg-negatives. This suggests the existence of poorly understood non-CM CrAg status-related morbidity worth further exploration.

Study limitations

Our review has several limitations. Most of the studies included in the review were observational with very few RCTs. The effect of pre-emptive fluconazole on the incidence of CM in CrAg-positive patients was evaluated indirectly even in the included RCTs because none of the studies compared pre-emptive fluconazole to no fluconazole or to an alternative pre-emptive therapy in CrAg-positive patients. Consequently, we report only indirect evidence

for the effectiveness of the WHO CrAg screen-and-treat strategy. Furthermore, not all studies evaluated our predefined main outcomes of interest. This led to variability in the denominators (number of studies and number of participants) across the outcomes. Also, the data were scarce for several outcomes, with zero cells leading to unstable estimates with wide confidence intervals.

Authors conclusion

Targeted fluconazole pre-emptive therapy offered to CrAg-positive patients dramatically reduces the risk of incident CM compared to no fluconazole but has unclear effects on all-cause mortality. The high prevalence of asymptomatic CM in CrAg-positive patients together with low lumbar puncture uptake, justifies the need for reliable point-of-care tests capable of properly identifying CrAg-positive patients at higher risk of underlying asymptomatic CM. The availability of sustainable fluconazole in ART programs is essential for effective pre-emptive strategies.

TABLES AND FIGURES

Table 1. Characteristics of included studies

Author, Year	Study design	CrAg test	Follow-up	Country	N*	Pre-emptive fluconazole therapy	Outcomes assessed			
							CrAg positivity	Asymptomatic CM in CrAg+	CM during follow-up	Mortality during follow-up
Desmet, 1989	Prospective cohort	LA	None	Democratic Republic of Congo	450	No	X	X		
Liechty, 2007	Retrospective screening of stored sera	LA	> 3 months	Uganda	377	No	X		X	X
Jarvis, 2009	Retrospective screening of stored sera	LA	1 year	South Africa	707	No	X		X	X
Meya, 2010	Prospective cohort	LA	47 months (median)	Uganda	295	200 - 400 mg/day for 2 - 4 weeks	X		X	X
Pongsai, 2010	Retrospective cohort	LA	1 year	Thailand	85	Yes (dose not reported)	X	X	X	
Mamoojee, 2011	Retrospective screening of stored sera	LA	Not reported	Ghana	92	No	X			
Linares, 2012	Retrospective screening of stored sera	LFA	1 year	Peru	365	No	X		X	X
Osazuwa, 2012	Cross-sectional study	LA	None	Nigeria	81	No	X			
Smith, 2013	Retrospective screening of stored sera	LFA	None	Vietnam	226	No	X			

Ganiem, 2014	Retrospective screening of stored sera	LFA	Not reported (however, for each included patient, follow up was from diagnosis of HIV till incidence of death)	Indonesia	810	No (blanket primary prophylaxis to patients with < 200 CD ₄ cell count)	X			X
Mckenney, 2014	Retrospective screening of stored sera	LFA	Not reported	USA	1,872	Not reported	X			
Manabe, 2015	Prospective cohort	LA	> 1 year	USA	117	Yes (dose and duration at physician's discretion)	X		X	X
Pac, 2015	Prospective cohort	LA	6 months	Uganda	177	800 mg/day for four weeks	X	X	X	X
Kapoor, 2015	Prospective cohort	LFA	6 months	Tanzania	216	800 mg/day for two weeks, then 400mg/day for two weeks	X		X	X
Mfinanga, 2015	Prospective screening in the context of a randomised trial	LFA	1 year	Tanzania and Zambia	717	800 mg/day for two weeks, then 400 mg/day for 8 weeks followed by 200 mg/day till CD ₄ above 200 cells/ μ l	X	X		X
Chipungu, 2015	Prospective cohort	LFA	6 months	Malawi	113	800 mg/day for two weeks, then 400 mg/day	X		X	

						for 8 weeks followed by 200 mg/day till CD4 above 200 cells/ μ l				
Vallabhaneni, 2015	Retrospective cohort	LA	1 year	South Africa	1,170	Yes (dose and duration at physician's discretion)	X		X	
Ezeanolue, 2016	Retrospective? Screening of stored whole blood samples	LFA	Not reported	Nigeria	2,752	No	X			
Longley, 2016	Prospective cohort	LFA	1 year	South Africa	645	800 mg/day for two weeks, then 400 mg/day for 8 weeks followed by 200 mg/day till CD4 above 200 cells/ μ l	X	X	X	X
Morawski, 2016	Prospective cohort	LFA	1 year	Uganda	2,135	800 mg/day for two weeks, then 400 mg/day for 8 weeks followed by 200 mg/day till CD4 above 200 cells/ μ l	X		X	X

Abbreviations: CrAg = cryptococcal antigen; LA = latex agglutination; LFA = lateral flow assay

*N is number of patients except for Mckenney and Ezeanolue (number of stored samples)

Table 2. Incidence of cryptococcal meningitis during follow-up

			Incidence of CM during follow-up, % (95%CI)		Risk ratio (95% CI)	p-value
			CrAg-positives	CrAg-negatives		
Interventions offered to CrAg-positive participants	Number of studies	Number of participants (N)				
No pre-emptive fluconazole	3	1,057	21.4 (11.6 – 34.4)	0.4 (0.1 – 1.0)	52.7 (6.4 – 431.2)	0.0002
Any pre-emptive fluconazole	9	4,708	6.6 (3.8 – 10.5)	0.3 (0.2 – 0.5)	15.6 (4.5 – 53.8)	0.0001
Stratified analysis						
Pre-emptive fluconazole initiated at <800 mg/day	4	1,635	9.1 (2.5 – 21.7)	0.6 (0.3 – 1.0)	15.9 (3.3 – 75.7)	0.0005
Pre-emptive fluconazole initiated at 800 mg/day	5	3,073	6.0 (3.2 – 10.3)	0.1 (0 – 0.3)	14.9 (2 – 111.7)	0.009
Pre-emptive fluconazole initiated at 800 mg/day after post-screening lumbar puncture	2	810	0 (0 – 0.1)	0.4 (0 – 1.0)	5.7 (0.7 – 49.8)	0.12

Abbreviations: CrAg, cryptococcal antigen; 95%CI, 95% confidence interval; CM, cryptococcal meningitis.

Table 3. All-cause mortality rates during follow-up

			All-cause mortality during follow-up, % (95%CI)		Risk ratio (95% CI)	p-value
			CrAg-positives	CrAg-negatives		
Interventions offered to CrAg-positive participants	Number of studies	Number of participants (N)				
No pre-emptive fluconazole	2	692	32.6 (19.1 – 48.5)	8.8 (6.7 – 11.2)	3.6 (2.2 – 5.8)	0.001
Any pre-emptive fluconazole	7	4,140	17.2 (12.8 – 22.2)	16.9 (15.8 – 18.2)	1.7 (0.7 – 4.2)	0.23
Stratified analysis						
Pre-emptive fluconazole initiated at <800 mg/day	2	395	25.9 (11.1 – 46.3)	6.5 (4.2 – 9.5)	9.4 (0.04 – 2069)	0.42
Pre-emptive fluconazole initiated at 800 mg/day	5	3,745	16.2 (11.8 – 21.5)	18 (16.8 – 19.4)	1.3 (0.6 – 3.2)	0.52
Pre-emptive fluconazole initiated at 800 mg/day after post screening lumbar puncture	3	1,538	25 (15.5 – 36.6)	11.8 (10.1 – 13.6)	2.3 (1.5 – 3.4)	0.001

Abbreviations: CrAg, cryptococcal antigen; 95%CI, 95% confidence interval.

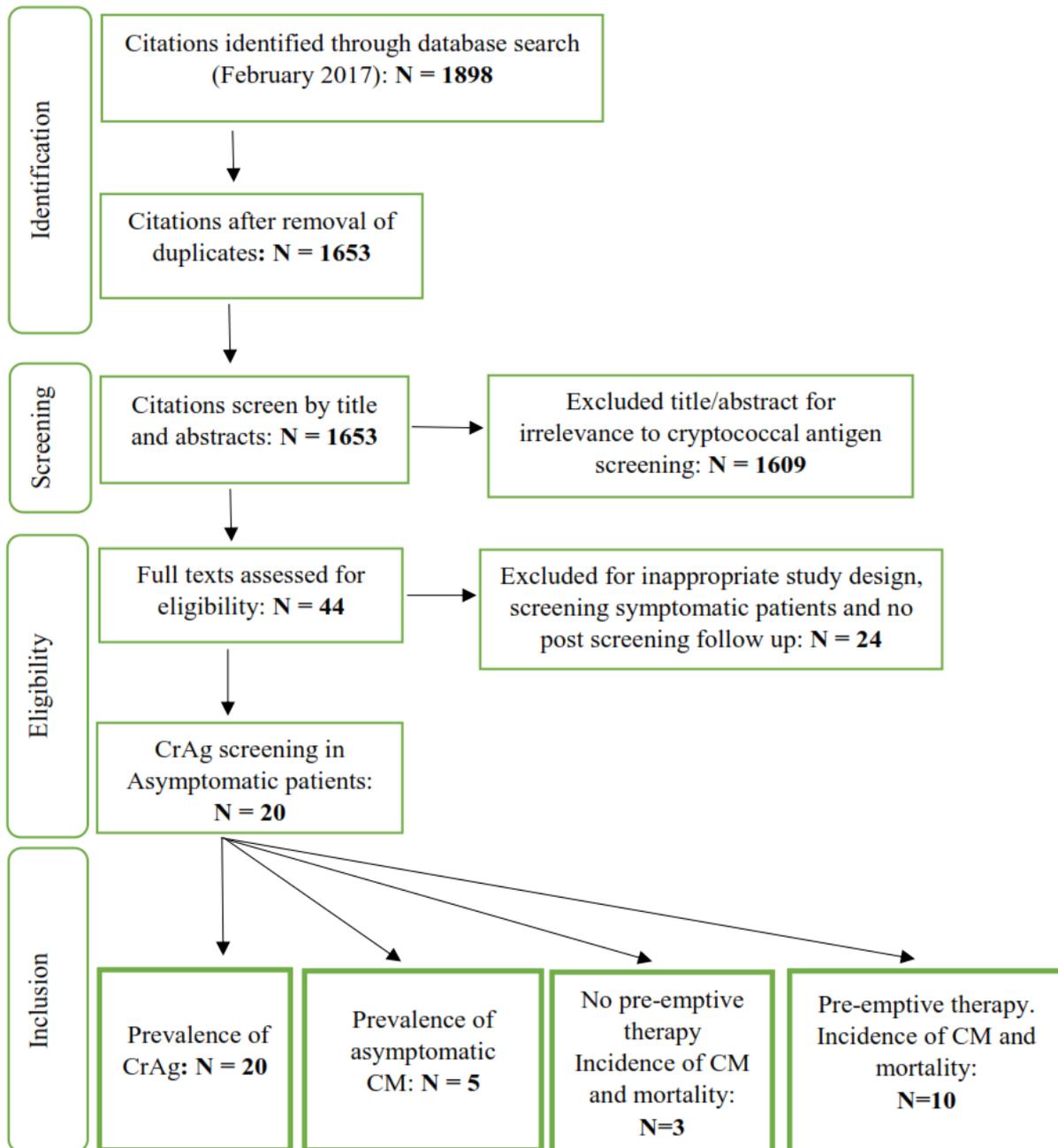


Figure 1. Flow diagram of studies in the review

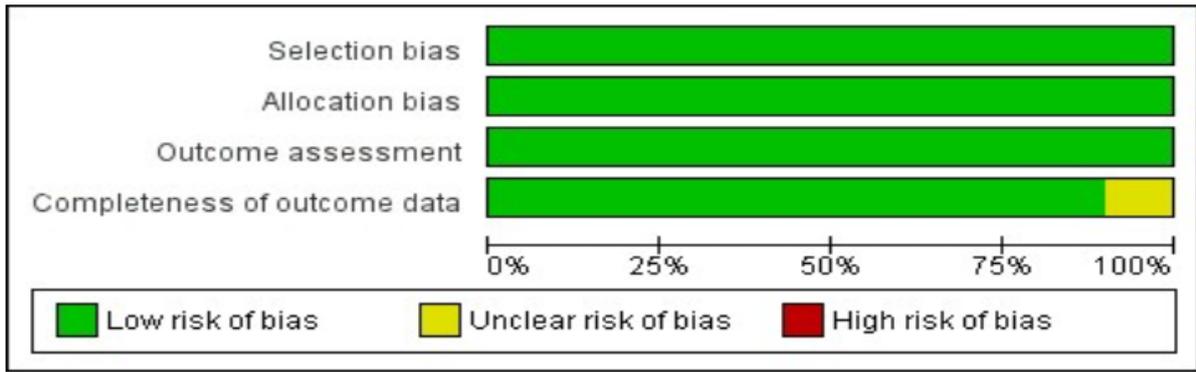
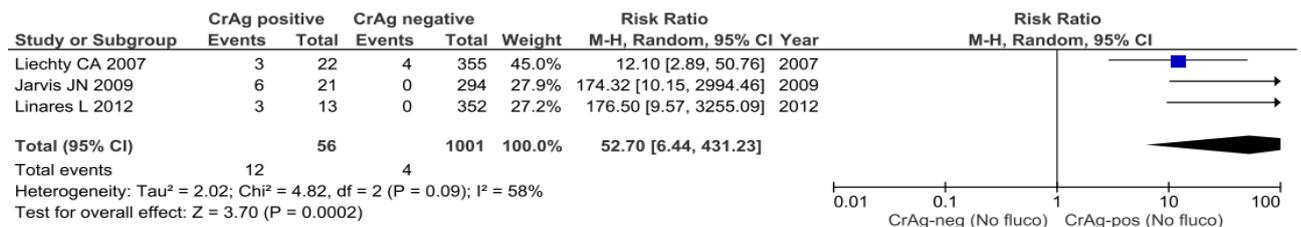


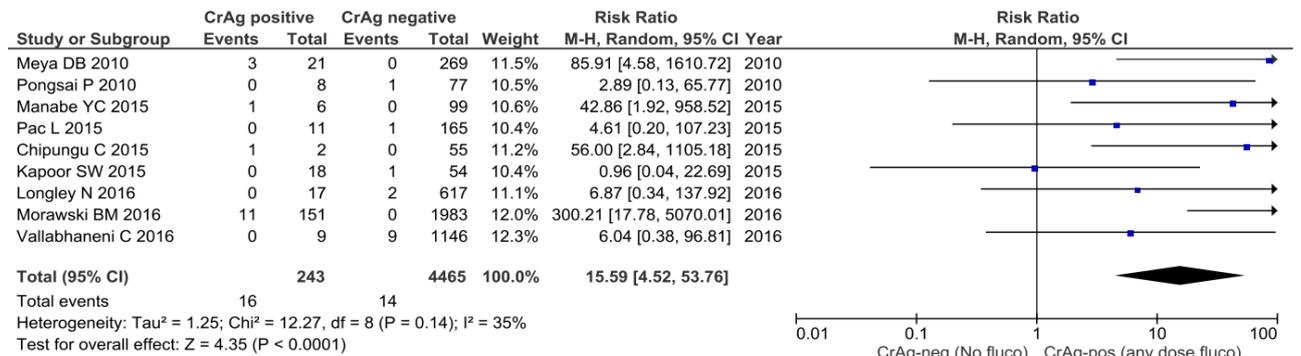
Figure 2. Risk of bias graph: review authors' judgements about each risk of bias item presented as percentages across all included studies.

	Selection bias	Allocation bias	Outcome assessment	Completeness of outcome data
Chipungu C 2015	+	+	+	+
Kapoor SW 2015	+	+	+	+
Longley N 2016	+	+	+	+
Manabe YC 2015	+	+	+	+
Meya DB 2010	+	+	+	+
Mfinanga S 2015	+	+	+	+
Morawski BM 2016	+	+	+	?
Pac L 2015	+	+	+	+
Pongsai P 2010	+	+	+	+
Vallabhaneni C 2016	+	+	+	+

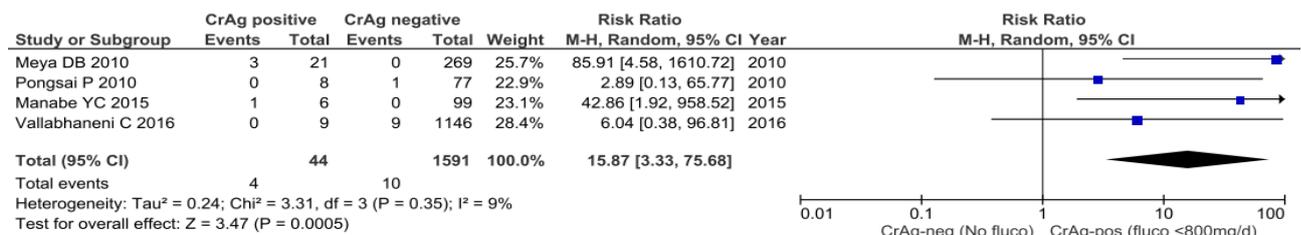
Figure 3. Risk of bias summary: review authors' judgements about each risk of bias item for each included study



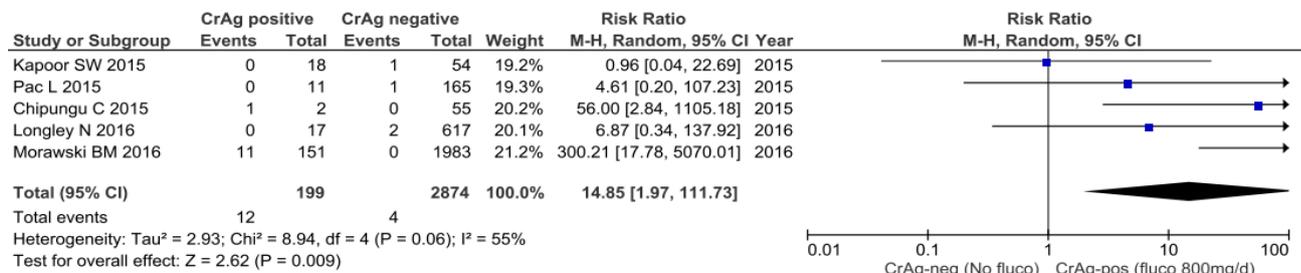
A. No fluconazole to CrAg-positive patients



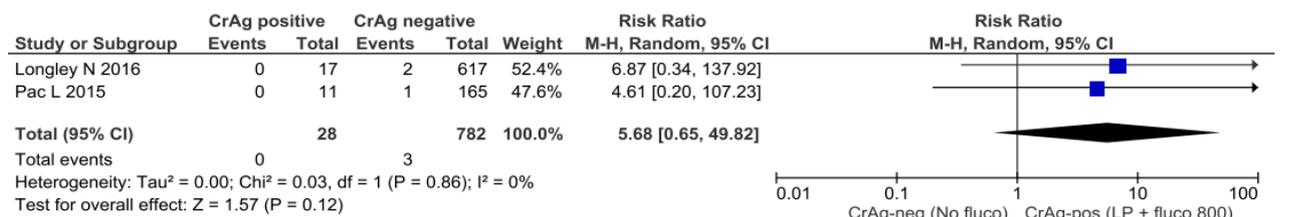
B. Fluconazole pre-emptive therapy initiated at any dose



C. Fluconazole pre-emptive therapy initiated at less than 800 mg/day

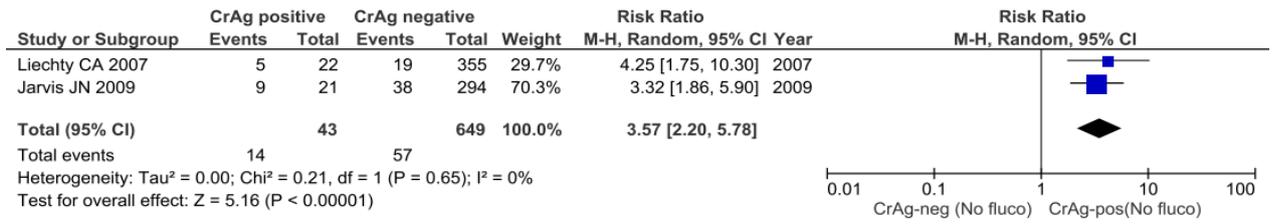


D. Fluconazole pre-emptive therapy initiated at 800 mg/day

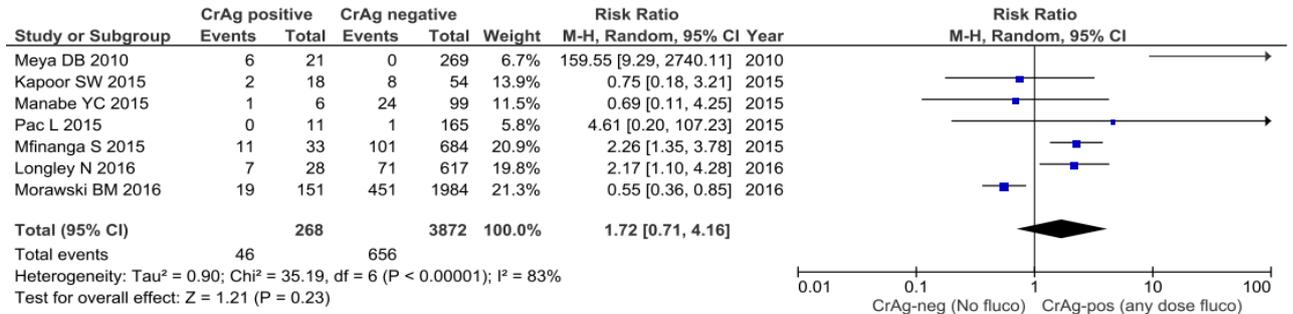


E. Fluconazole pre-emptive therapy initiated at 800 mg/day after post screening lumbar puncture to CrAg-positives

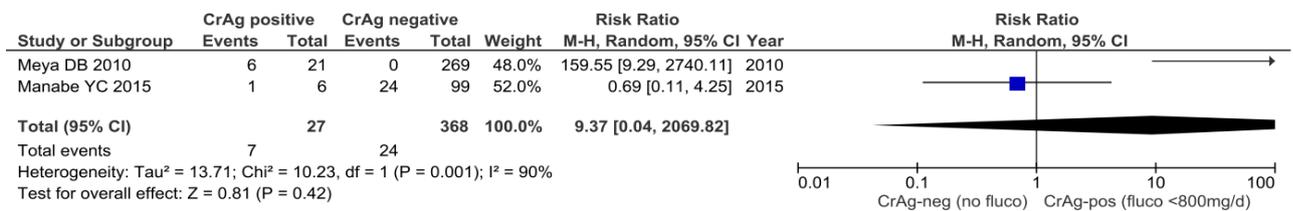
Figure 4. Incidence of cryptococcal meningitis during follow-up



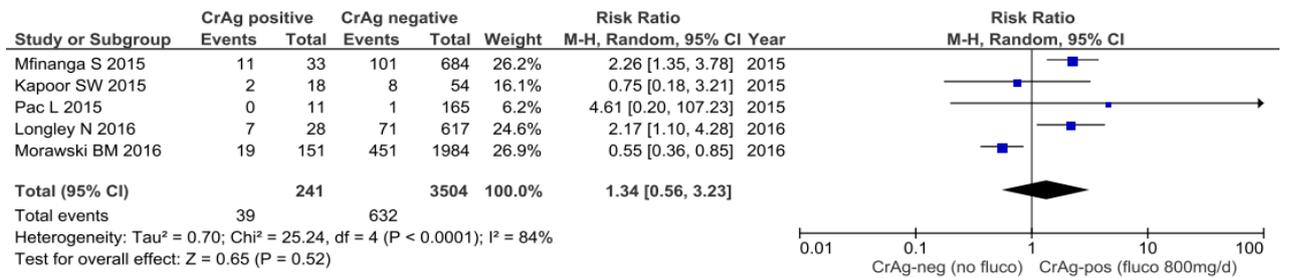
A. No fluconazole to CrAg positive patients



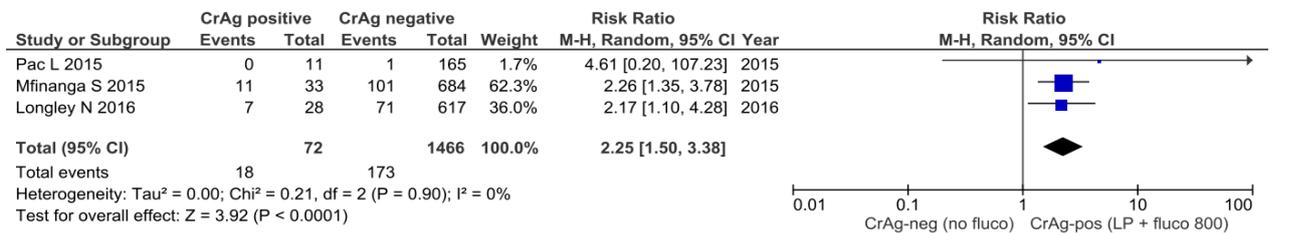
B. Fluconazole pre-emptive therapy initiated at any dose



C. Fluconazole pre-emptive therapy initiated at less than 800 mg/day



D. Fluconazole pre-emptive therapy initiated at 800 mg/day



E. Fluconazole pre-emptive therapy initiated at 800 mg/day after post screening lumbar puncture to CrAg-positives

Figure 5. All-cause mortality during follow-up

APPENDICES

Appendix 1: MEDLINE search strategy

Ovid MEDLINE(R) Epub Ahead of Print, In-Process & Other Non-Indexed Citations, Ovid MEDLINE(R)

Searches

- exp "Antigens, Fungal"/ or exp "Clinical Laboratory Techniques"/ or exp "Point-of-Care Systems"/ or exp "Latex Fixation Tests"/ or exp "Enzyme-Linked Immunosorbent Assay"/ or exp "Immunoenzyme Techniques"/mt or exp "Immunologic Tests"/ or exp Immunoassay/ or "Immunoenzyme Techniques"/ or exp Reagent Kits, Diagnostic/ or Immunochromatography/ or (Dipstick or RDT or (Diagnos* adj2 kit) or (antigen adj (test or detection)) or (reagent adj2 strip*) or (Immuno* adj2 test) or Point-of-Care Systems or ((simple or easy or quick or rapid) adj4 (test* or diagnos* or kit or kits)) or "latex agglutination" or "Latex particle agglutination" or "enzyme immunoassay*" or "enzyme immuno-assay" or "enzyme-linked immunoassay*" or ELISA* or EIA or
- 1 immunochromatograph* or lateral-flow or LFA or Crag).ti,ab,kf.
- exp "Meningitis, cryptococcal"/ or exp cryptococcosis/ or exp "Cryptococcus neoformans"/
- 2 or (toruloma* or cryptococc*).ti,ab,kf.
- exp "HIV Infections"/ or exp HIV/ or "sexually transmitted diseases, Viral"/ or (hiv* or "human immunodeficiency virus" or "human immunodeficiency virus" or "human immunodeficiency virus" or "human immune-deficiency virus" or ("human immun*" adj2 "deficiency virus") or "acquired immunodeficiency syndrome" or "acquired immunodeficiency syndrome" or "acquired immuno-deficiency syndrome" or "acquired immune-deficiency syndrome" or ("acquired immun*" adj2 "deficiency syndrome")).ti,ab,kf.
- 3
- 4 1 and 2 and 3
- 5 limit 4 to yr="1981 -Current"

Appendix 2: Joanna Briggs institute cohort study quality assessment tool (tailored to the review question)

Response to each question is either Yes, No, Unclear or not applicable (N/A)

Selection bias

1. Were the two groups (CrAg positive and negative) recruited from the same population of patients with <100 CD4 count?

Allocation bias

1. Was CrAg screening done using the same tests in the whole study population?
2. Was CrAg screening done using either Latex agglutination test or IMMY LFA?
3. Were the two groups (CrAg positive and negative) similar in baseline characteristics and other co-morbidities (confounders)?
4. If both groups had some identified differences, were strategies to deal with these differences stated in the study (stratification and adjustment during analysis)

Outcome assessment

1. Was there baseline diagnosis of CM through lumbar punctures after screening to ensure both groups did not have the primary outcome of interest?
2. During follow up, how was the incidence of CM ascertained? Was it by Indian ink stain or culture or CrAg in CSF?

Completeness of Outcome data

1. Was the follow up time reported and sufficient to be long enough for outcome to occur (minimum 6 months for this review)
2. Was follow-up complete and if not, were the reasons to loss to follow up described and explored?
3. Were strategies to address incomplete follow-up utilised (for example incidence expressed in person-years at risk?)

Appendix 3: Risk of bias evaluation tool adapted from Joanna Briggs Institute checklist for cohort studies

Overall, the methodological quality of the 10 studies included in the analysis of the effectiveness of pre-emptive fluconazole therapy is summarised in Figure 2 and the quality assessment result of each study in Figure 3. The median sample size was 256 patients (IQR: 107 – 717). In these studies, when patient with >100 CD4 cells/ μ l cells were also screened, only CrAg-positive patients with <100 CD4 cells/ μ l were offered pre-emptive fluconazole. In 4 (40%) of the studies, CrAg-positive patients were consented for post-CrAg screening lumbar puncture and in all studies, pre-emptive fluconazole was offered only to asymptomatic patients as clearly described in each study. In one study where 5 CrAg-positive patients were not offered pre-emptive therapy, their outcome was clearly described and not included in the analysis of those who took pre-emptive fluconazole [105]. During the follow-up period, 9 (90%) of the studies reported incidence of first episode of post-ART CM in both CrAg-positive and -negative patients but only 7 (70%) reported the incidence of mortality. However, in one study, it was not very clear how reporting was done in CrAg-negative patients [30] (Figures 2 and 3). In 2 (20%) studies, lost to follow up was considered and reported.

All patients (CrAg-positives and -negatives) were placed on ART (deferred by 2 – 4 weeks in CrAg-positive patients) and followed up for a median duration of 1 year (IQR: 0.5 – 1). Adherence to ART and fluconazole was not evaluated in this review because they were infrequently reported in the included studies.

2.2. Cryptococcal antigen detection and prevention in Cameroon

2.2.1. Summary

Background

Cryptococcal meningitis (CM) is a major cause of AIDS-related mortality in Africa. Detection of serum cryptococcal antigen (CrAg) predicts development of CM in antiretroviral (ART) naïve advanced HIV-infected patients. Systematic pre-ART CrAg screening and pre-emptive oral fluconazole is thus recommended.

Methods

ART-naïve adult outpatients with <100 CD₄ cells/mm³ presenting to the Yaoundé Central Hospital, Cameroon were screened for CrAg using the IMMY lateral flow assay (LFA). CrAg positive patients with proven CM were treated with combination antifungal therapy and asymptomatic cases offered fluconazole. Simultaneous on-site evaluation of CrAg detection using the new LFA Biosynex CryptoPS® test was performed and both tests were subsequently compared to a commercialized CrAg enzyme immunoassay (EIA).

Results

Prevalence of serum CrAg in 186 screened adults was 7.5% (95%CI: 4.5 – 12.4). In CrAg positive patients, CM prevalence was 45.5% (95%CI: 18.3 – 75.7). IMMY and Biosynex CryptoPS strongly agreed in serum, plasma, and cerebrospinal fluid (Kappa: 98.4%, 99.5%, 100%, respectively, $p<0.001$). Compared to EIA as a reference standard, serum specificities were 96.6% and 98.3%, respectively. With Biosynex CryptoPS, all CM patients were serum T2-band positive compared to none without CM. Median EIA titre was 160 (IQR: 13.5 – 718.8) and strongly correlated with Biosynex CryptoPS T2-band positivity. Overall, incidence of all-cause mortality was 31.5 per 100 person-years-at-risk (95%CI: 23.0 – 43.1). No incident case of CM occurred during 1-year follow-up.

Conclusion

HIV-associated latent cryptococcosis is common in Cameroon, warranting integrated systematic screening and treatment. Biosynex CryptoPS holds promise for rapidly stratifying CrAg positive patients for optimal management.

2.2.2. Draft article 2: In revision in *Frontiers in Microbiology*

Title: Cryptococcal antigen screening in Cameroon and contribution of a new semi-quantitative Biosynex CryptoPS test: ANRS 12312 PreCASA study.

Short title: Cryptococcal antigen screening in AIDS in Cameroon

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Word counts

Abstract: 249 words, Main text: 2942 words

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Note

This study was presented in part at the 10th International conference on Cryptococcus and Cryptococcosis (ICCC) in Iguazu in Brazil from 26th – 30 March 2016 and won an oral presentation prize.

Abstract

Objective

To determine the prevalence of cryptococcal antigen (CrAg) in antiretroviral therapy (ART) naïve adults with advanced HIV-infection and the impact of pre-emptive oral fluconazole on the incidence of cryptococcal meningitis (CM) during the first year of ART

Design

Prospective cohort

Methods

ART-naïve adult outpatients with <100 CD₄ cells/mm³ presenting to the Yaoundé Central Hospital, Cameroon were screened for CrAg using the IMMY lateral flow assay (LFA). CrAg positive patients with proven CM were treated with combination antifungal therapy and asymptomatic cases offered fluconazole. Simultaneous on-site evaluation of CrAg detection using the new LFA Biosynex CryptoPS® test was performed and both tests were subsequently compared to a commercialized CrAg enzyme immunoassay (EIA).

Results

Prevalence of serum CrAg in 186 screened adults was 7.5% (95%CI: 4.5 – 12.4). In CrAg positive patients, CM prevalence was 45.5% (95%CI: 18.3 – 75.7). IMMY and Biosynex CryptoPS strongly agreed in serum, plasma, and cerebrospinal fluid (Kappa: 98.4%, 99.5%, 100%, respectively, $p<0.001$). Compared to EIA as a reference standard, serum specificities were 96.6% and 98.3%, respectively. With Biosynex CryptoPS, all CM patients were serum T2-band positive compared to none without CM. Median EIA titre was 160 (IQR: 13.5 – 718.8) and strongly correlated with Biosynex CryptoPS T2-band positivity. Overall, incidence of all-

cause mortality was 31.5 per 100 person-years-at-risk (95%CI: 23.0 – 43.1). No incident case of CM occurred during 1-year follow-up.

Conclusion

HIV-associated latent cryptococcosis is common in Cameroon, warranting integrated systematic screening and treatment. Biosynex CryptoPS holds promise for rapidly stratifying CrAg positive patients for optimal management.

Key words: Cryptococcosis, cryptococcal antigen, screening, point of care, lateral flow assay.

Introduction

Cryptococcal meningitis (CM) is a major cause of morbidity and mortality among patients with advanced HIV infection especially in resource limited settings. Cryptococcosis is responsible for up to 15% of AIDS related mortality of which 75% is in Sub-Saharan Africa [1]. Global estimates suggest that CM represents the leading cause of adult meningitis worldwide and the second cause of death after tuberculosis [2, 3]. Though existing evidence of an association between antiretroviral therapy (ART) program implementation and decreased CM incidence [4, 5], in spite of efforts to scale up ART coverage, at least 20% of patients still present to ART care with < 100 CD₄ cells/ml [6]. More so, in the African setting [1], in the latter patients with detectable untreated blood cryptococcal antigen (CrAg), about 25% subsequently develop CM within the first year of ART compared to as few as no cases in CrAg negatives [7].

In this setting, two strategies to reduce the incidence of CM have been suggested. Universal fluconazole-based primary prophylaxis, which resulted in decreased CM incidence, but had no impact on overall mortality and thus not widely adopted [8, 9]. The second strategy consisted of pre-ART systematic screening for CrAg in blood in profoundly immune depressed patients followed by pre-emptive administration of fluconazole to asymptomatic CrAg positive patients [10, 11]. The latter was endorsed by 2011 World Health Organisation (WHO) rapid advice [12] which recommended screening either with latex agglutination (LA) test or the FDA-approved lateral flow assay (LFA) point of care (POC) immunochromatographic test (IMMY® diagnostics, Norman, Oklahoma, USA) [13, 14]. This approach which has shown survival benefits when coupled with enhanced adherence to ART [1, 15-17], is cost-effective [15, 18-20] and has been incorporated into national guidelines of many countries in the Southern part of Africa [1]. Nevertheless, Central Africa lags in adopting this strategy. Consequently, through this prospective cohort study in Cameroon, we aimed to determine the prevalence of CrAg in blood and asymptomatic CM in ambulatory ART-naïve adults presenting with < 100 CD₄

cells/ml using IMMY LFA and the incidence of CM during first year of ART in the context of screening. A second objective was to assess the performances of a recently-developed semi-quantitative immunochromatographic POC test (Biosynex CryptoPS, BIOSYNEX® diagnostics, Strasbourg, France) and ultimately compared the performances of both POC tests to those of the commercialised enzyme immunoassay (EIA), the Premier® CrAg (Meridian Bioscience, Inc, Ohio, USA), considered in this study as the reference standard.

Methods

Study setting

This study was carried out at the Day Hospital of the Yaoundé Central Hospital in Cameroon where the French National Agency for Research on HIV and Hepatitis (ANRS) centre is located. It is a major HIV treatment centre which follows up over 9,000 patients presently on ART using updated national guidelines following WHO recommendations [21].

Study procedure

Ethical clearance was obtained from the National Ethics Committee for Research in Human Health, Cameroon. HIV-infected, ART naïve ambulatory adults (> 18 years) presenting with <100 CD₄ cells/ml, with no history of cryptococcal meningitis, consenting to participate in the study, were included. A standardized questionnaire was used to anonymously record baseline sociodemographic data as well as current symptoms at each visit.

On inclusion, participants were checked for symptoms evocative of CM, and those with recent history of altered mental status or febrile seizures were excluded. In the absence of symptoms, specimens (blood and urine) were collected for same day CrAg screening. CrAg screening was performed in all patients on fresh serum and urine samples using the IMMY LFA [14] according to manufacturer's procedure. In addition, all samples were also tested and interpreted

using a newly developed LFA-based immunochromatographic test called Biosynex CryptoPS without prior knowledge of the results obtained with the IMMY LFA test.

Briefly, Biosynex CryptoPS can detect the four serotypes of *Cryptococcus* spp., and provides results within 10 minutes. It includes two bands: the T1-band is qualitative and the T2-band is semi-quantitative. The T2 band will only appear in patients with elevated CrAg titres [22]. Additionally, plasma samples obtained on the same day as the serum were also screened with both POC.

Patients were considered CrAg positive if serum samples were positive using the IMMY LFA test but all patients with detectable CrAg in any fluid were consented for lumbar puncture (LP). Freshly obtained cerebrospinal fluid (CSF) was tested with both POC tests. Direct examination was performed (Indian ink staining) and CSF was cultured on Sabouraud dextrose agar. Participants with laboratory proven CM (positive India ink and/or culture) were offered treatment within the phase III ACTA trial [23].

Participants with detectable CrAg but no laboratory proven CM were placed on pre-emptive oral fluconazole: 800mg/day for two weeks, then 400mg/day for 8 weeks and 200mg/day from week 10 till two consecutive control CD4 cell counts at month 6 and 12 were both above 200 cells/ml [12]. CrAg negative participants were immediately started on ART without fluconazole, while those on fluconazole and those treated for CM, had ART deferred by 2 weeks and 4 weeks, respectively.

Follow up visits were every two weeks for the first ten weeks, then every three months for one year. At each visit, symptoms of CM were carefully searched and if clinical suspicion, LP was performed. ART adherence evaluation and support through counselling and medication dispensation were also done. In case of missed appointments, participants or closed family members were followed-up by phone and verbal autopsies conducted for participants who died.

To assess the performances of the LFA tests, the EIA Premier® CrAg test (Meridian Bioscience, Inc, Ohio, USA) was chosen as reference standard in this study. Aliquots of the 186 serum samples stored at -80°C at the Centre Pasteur du Cameroun were subsequently transported frozen under rigorous conditions to France for CrAg testing using EIA Premier® according to manufacturer's procedure. For all EIA CrAg positive specimens, CrAg titre determination was performed by EIA and with both POC tests (with Biosynex CryptoPS, both T1 and T2 titres were determined). Results were expressed as reciprocal titre.

Statistical analysis

Data were analysed using STATA 13.0 software (Statacorp, Texas, USA). Results of CrAg screening in the population were expressed as percentages (with their 95% confidence interval [CI]). Categorical variables were compared using Pearson's Chi-square or Fischer's exact test as required. Continuous variables were reported as means (and standard deviation [SD]) or median (and interquartile range [IQR]) as required. Student t-test or Wilcoxon rank sum test were respectively used to compare means or medians as required.

Agreement between IMMY and Biosynex CryptoPS tests in classifying study participants as positive or negative in each specimen type with Indian ink staining or culture of CSF were appraised using Kappa statistics and reported as percentages.

With reference to EIA, two-by-two tables were used to estimate sensitivity and specificity of both POC tests and reported as percentages with their 95%CI.

Cox proportional hazard ratios (HR) and Kaplan-Meier curves were used to compare survival differences during the first year following CrAg screening. In the multivariate analysis, mortality-associated factors with p-value <0.1 in the univariate analysis were included in the final model and reported as adjusted hazard ratios (aHR) with their 95%CI. Evidence against the null hypothesis was considered for a two-tailed p-value of <0.05.

Results

Prevalence of latent cryptococcosis and CM based on screening using IMMY LFA

Between July 2015 and March 2017, 186 patients were screened for CrAg. Mean age was 38.2 years (SD: 10.0) and 67.7% (126/186) were women. Median BMI was 21.0 kg/m² (IQR: 18.8 – 23.2) and median CD₄ was 44 cells/ml (IQR: 27 – 75) with no difference between those CrAg positive and negative (Table 1).

Prevalence of serum CrAg was 7.5% (14/186) (Table 2). Considering all screened specimens (serum/plasma, and urine), 23.1% (43/186) had detectable CrAg (Figure 1). Among the 43 patients with detectable CrAg, 27 (62.8%) accepted a LP including 11 of the 14 patients with positive serum CrAg. Four of the 27 LPs did not yield CSF (4 patients with CrAg detection positive in urine only) and 21/23 CSF (91.3%) were cultured (1 positive Indian ink CSF and one isolated urine CrAg were not cultured).

Overall, CM diagnosed in 21.7% (5/23) of patients by means of a positive Indian ink staining (5/5), positive detection of CrAg (4/5) and positive culture (4/4) (Table 3). Clinically, patients with positive serum CrAg detection and CM were not different from those without CM (Table 3). Moreover, 3/5 had no signs suggestive of meningitis. None of the 29 patients with isolated positive urine CrAg had CM.

Subsequent follow-up and outcome in the study population

All 5 patients with CM were enrolled in the ACTA trial and all the others with detectable CrAg by IMMY LFA considered as latent cryptococcosis, were prescribed fluconazole. Median follow-up for the study population was 323 days (IQR: 92 – 363), not different in serum CrAg positive and negative patients. Median ART initiation was 3 days (IQR: 1 – 7) in CrAg negative patients and 97.5% of all patients started first line regimen containing tenofovir-lamivudine-efavirenz. Overall adherence to ART and fluconazole was 100%. At 6 and 12 months, median

CD₄ cell/ml were respectively 208 (IQR: 129 – 284) and 256 (IQR: 183 – 315), similar in CrAg positive and negative patients (p=0.59). There was no incident case of cryptococcal meningitis during the total 123.9 person-years-at-risk (PYAR) follow-up time.

Overall, all-cause mortality was 21% (39/186) at a cumulative incidence rate of 31.5 per 100 PYAR, occurring within a median follow-up period of 82 days (IQR: 33 – 194), 71.8% (28/39) within the first 3 months of ART. Serum CrAg positive patients were less likely to survive than those who were CrAg negative, p=0.09 (Figure 3). In those with baseline confirmed CM, mortality in the first year was 60% (3/5), one death was due to CM (supplementary table) on day 4 of treatment.

In the study population, the presence of symptoms of respiratory and gastrointestinal infections during follow-up were significantly associated with increased mortality and remained strongly associated with death after adjusting for other factors in the multivariate analysis while baseline CrAg positivity was not (Table 4).

Impact of the test and the specimen used for CrAg screening

With Biosynex CryptoPS, looking at the T1-band results, 5.9% of patients (11/186) were positive in serum (corresponding to 11/14 of the serum positive with IMMY LFA), 5.9% in urine (11/186, corresponding to 11/42 of the urine positive with IMMY LFA) and 17.4% (4/23) in CSF (4/4 of those positive with IMMY LFA) (Table 2). No patient was detected with isolated urine CrAg. Of the 11 patients with the positive T1-band in serum, 10 had CrAg positive in urine, and 45.5% (5/11) were also T2-band positive. All five patients with positive T2-band had CM while none with negative T2-band had. Of note, 2 of the 5 with CM had T2-band positive in urine.

Concerning the classification of specimens as positive or negative, IMMY LFA and Biosynex CryptoPS agreed in 98.4% of serum and 100% of CSF (Table 2) but in urine, 15.6% (29/186)

of patients had isolated urine CrAg with the IMMY LFA (Figure 1). Each POC test had a 98.9% agreement in classifying plasma and serum specimens from the same patient as positive or negative (data not shown).

Of the 186 sera tested with EIA CrAg, 4.3% (8/186) were positive. No serum previously categorised as negative by both POC test was found positive by EIA, including those with isolated urine CrAg by IMMY LFA. Compared to EIA as reference standard, IMMY LFA and Biosynex CryptoPS both had a sensitivity of 100% and respective specificities of 96.6% (95%CI: 92.8 – 98.6) and 98.3% (95%CI: 95.2 – 99.7).

Median EIA CrAg reciprocal titre in serum was 160 (IQR: 13.5 – 718.8), higher in those with microbiologically confirmed CM: 525 (IQR: 160.5 – 912.5) than in those without 6.4 (IQR: 1 – 20.5), $p=0.003$. EIA reciprocal titre threshold of 160 was associated with the presence of confirmed CM ($p=0.03$). Both POC tests CrAg titres agreed with EIA titres in stratifying patients with elevated titres but the T2-band of the Biosynex CryptoPS showed a stronger pattern of agreement (Figure 2). Of note, among the 6 sera that were initially positive by IMMY LFA, 3 had titres that ranged from 10 to 20 with the same POC test, among those 3 initially positive by Biosynex CryptoPS, 2 had titres of 10 and 80 and both POC agreed on one sample that had titre of 1:10.

Considering serum CrAg positivity as a surrogate to confirmatory CM diagnosis, the IMMY LFA predicted 45.5% (5/11) of asymptomatic CM while the Biosynex CryptoPS T1-band predicted 55.5% (5/9) and the T2-band predicted 100% (5/5).

Discussion

In Cameroon where 4.3% [24] of adults are HIV-infected, we found in those presenting with <100 CD₄ cells/ml, that 7.5% were serum CrAg positive using the IMMY LFA (5.9% and 4.3% using Biosynex CryptoPS and EIA, respectively) indicating a high risk of CM warranting

implementation of systematic pre-ART CrAg screening in this population [12]. Nevertheless, doing that in a local context where current application of the HIV “test-and-treat” strategy [21] does not recommend baseline pre-ART CD4 T-cell count remains challenging.

Depending on the test used for screening, one out of two to three patients with positive serum CrAg had asymptomatic CM, a finding like in other cohorts in the southern part of Africa [17, 25], suggesting that systematic LPs should be performed for all asymptomatic CrAg positive patients as a strategy to timely diagnose CM. Though we had high LP acceptability within the study, it is not always the case in routine settings with similar CM risks [17, 25], thus the need for more acceptable methods to timely diagnose CM [26]. This could be achieved by offering LPs only to those with higher CrAg titres, as high blood titres are associated with presence of CM [16, 25]. Identifying those with high titres could be done either through serial dilutions of CrAg positive samples [25, 27] or through the availability of a CrAg test capable of directly identifying those with high titres [22, 26]. Serial dilution is expensive, technically demanding, particularly for low-resource areas, requires a laboratory setting and prolongs diagnostic time. In our study, the Biosynex CryptoPS semi-quantitative T2-band, identified patients with high titres and these high titres correlated with laboratory evidence of asymptomatic CM [25]. Such promising performance could therefore be invaluable in CM diagnostic algorithms especially in settings with low LP uptake.

During the follow up period, there was no incident case of CM, a finding comparable to that of a South African cohort [25] where pre-emptive treatment was administered at the same tapering doses [12]. In rural Uganda, using 800mg/day pre-emptively for only four weeks [28] yielded similar results within six months of follow up. However, another recent Ugandan cohort described a failure of fluconazole administered at this recommended pre-emptive dose in preventing incident CM (HR:5.4) and death (HR: 3.2) within 6 months of screening and this failure was attributed to baseline CrAg titres $\geq 1:160$ [29]. Here, EIA titres of 1:160 was

associated with CM, thereby suggesting that patients of this Ugandan cohort at baseline might have had undiagnosed CM and that fluconazole at 800mg/day shown not to be suitable for inductive CM treatment [30] could be more appropriate as pre-emptive therapy. Overall, these data iterate the importance of considering antigen titre measurement as a proxy to timely CM diagnosis.

Overall mortality during first year of ART, though high, was comparable to findings in other cohorts in resource limited setting [31]. We found some evidence of CrAg positivity and risk of death but not as strong as elsewhere [2, 7, 25, 28], and this association disappeared upon adjusting with other factors [32]. Arguably, it could be due to our relatively smaller sample, but a plausible explanation could also be our approach of active CM case finding at baseline such that over time, risks of CM was similar in those found CrAg positive or negative. Notwithstanding, though mortality in CrAg negatives in our cohort was still unacceptably high, there were more early deaths in those screened as CrAg positive but only one was attributed to CM. CrAg positive patients may have other poorly understood factors that lead to death due to non-CM causes; or deaths may have been with undetected cryptococcosis. The precise cause of death is extremely hard to ascertain in this patient population.

Our study has some limitations. The number of patients screened for CrAg was lower than initially planned due to modifications in local guidelines on diagnosis and treatment of HIV which reduced the number of potentially eligible patients.

Nevertheless, we believe that our findings may facilitate management strategies of cryptococcosis in Sub-Saharan African countries based on excellent performances of both POC tests used. Considering EIA as the reference standard, both POC had excellent sensitivity while specificity was slightly better in serum for Biosynex CryptoPS. Of note, urines screening provided false positive results with the LFA IMMY as previously reported [25, 33]

Acknowledging the constraints around serial dilution for titre determination and considering that Biosynex CryptoPS T2-band directly uncovers those with high titres and CM, point out the urgent need for broad evaluation of this new test as a timely tool for asymptomatic CM diagnosis.

Conclusion

The prevalence of CrAg and asymptomatic CM in Cameroon is unexpectedly high, thereby suggesting that CM burden may also be comparably high as described in the Southern part of Africa. There is urgent need to integrate as part of HIV comprehensive care package, systematic CrAg screening and pre-emptive fluconazole therapy as a strategy to reduce HIV-associated CM morbidity and mortality. The Biosynex CryptoPS test is comparable to the IMMY LFA as POC CrAg screening test. Diagnostic lumbar punctures and/or effective antifungal therapy should be considered for patients screening positive with the Biosynex CryptoPS T2-band.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgement

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Table 1: Baseline characteristics of study population (n=186) categorised by serum CrAg status using IMMY LFA			
Variables at baseline	CrAg positive n=14	CrAg negative n=172	p- value
Socio-demographic			
Age in years, mean (SD)	39.3 (10.5)	38.5 (10.2)	0.55
Male, n (%)	5 (35.7)	55 (32)	0.77
Clinical			
History of tuberculosis, n (%)	0	9 (5.3)	0.99
History of liver disease, n (%)	0	5 (2.9)	0.99
Body mass index in Kgm², median (IQR)	20.6 (18.4 – 21.3)	21.0 (18.8 – 23.4)	0.36
Laboratory			
CD4 cells/ml, median (IQR)	44 (26 – 76)	48 (35 – 67)	0.48
Haemoglobin in g/dL, median (IQR)	10.5 (9.6 – 11.3)	9.9 (8.9 -11.3)	0.96
White blood cells x 10³/ml, median (IQR)	2 (2.0 – 5.0)	1 (1 – 3)	0.05
Alanine transaminase in IU/L, median (IQR)	32.4 (23.7 – 41.1)	27.6 (19.2 – 43.3)	0.96
Serum creatinine in g/dL, median (IQR)	1 (0.9 – 1.2)	1 (0.8 – 1.2)	0.95

Table 2: On site comparison of IMMY diagnostics LFA and Biosynex CryptoPS tests positivity in 186 fresh serum, plasma and urine samples and 23 cerebrospinal fluid (CSF) samples.

Specimen types	IMMY LFA test % (95% CI)	Biosynex CryptoPS % (95%CI)	% agreement (Kappa)	P-value
Serum	7.5	5.9	98.4	0.001
N = 186	(4.5 – 12.4)	(3.3 – 10.4)		
Plasma	6.5	5.9	99.5	0.001
N = 186	(3.7 – 11.1)	(3.3 – 10.4)		
Urine	22.6	5.9	83.3	0.001
N= 186	(17.1 – 29.2)	(3.3 – 10.4)		
CSF	17.4	17.4	100	0.001
N = 23	(6.2 – 40.3)	(6.2 – 40.3)		

Table 3: Baseline clinical and laboratory characteristics of patients screened serum CrAg positive using IMMY LFA and stratified by presence or absence of cryptococcal meningitis (CM)

Variables	With CM	Without CM	P-value
	n=5	n=18	
Symptoms			
Headache, n (%)	3 (60)	2 (11.8)	0.06
Fever, n (%)	1 (20)	3 (16.7)	0.99
Confusion, n (%)	1 (20)	2 (11.1)	0.54
Photophobia, n (%)	1 (20)	1 (5.6)	0.40
Clinical signs			
Neck stiffness, n (%)	2 (40)	0	0.04
Kernig sign, n (%)	2 (40)	0	0.04
Brudzinski sign, n (%)	2 (40)	1 (5.6)	0.10
CSF findings at inclusion			
IMMY LFA CrAg positive, n (%)	4 (80)	0	0.001
Indian ink positive, n (%)	5 (100)	0	0.001
Culture positive, n (%)	4 (80)	0	0.001
Glucose in g/L, median (IQR)	0.4 (0.4 – 0.5)	0.5 (0.40 – 0.5)	0.08
Proteins in g/L, median (IQR)	0.5 (0.4 – 0.8)	0.5 (0.3 – 0.7)	0.72

Table 4: Factors associated with mortality during the first year of ART in patients screened for CrAg

Factors	Univariable analysis			Multivariable analysis		
	HR	95% CI	P-value	aHR	95% CI	P-value
Presence of fever	4.2	1.5 – 12.1	0.007	3.8	1.3 – 11.2	0.02
Presence of respiratory symptoms	5.2	2.5 – 10.8	0.001	2.55	1.0 – 6.3	0.04
Presence of gastrointestinal symptoms	5.4	2.6 – 11.1	0.001	4.23	1.8 – 9.7	0.001
Baseline serum CrAg positive (IMMY Diagnostics)	2.2	0.9 – 5.7	0.09	1.73	0.5 – 6.2	0.4

**Supplementary Table: Probable causes of death in 39 patients during first year of ART
following CrAg screening**

Probable causes of death	CrAg positive	CrAg negative	Total deaths, n, (%)
Gastroenteritis	0	12	12 (30.8)
Respiratory/Tuberculosis	0	8	8 (20.5)
Unknown causes	2	6	8 (20.5)
Severe anaemia	0	0	3 (7.7)
Kaposi sarcoma	0	2	2 (5.2)
Cryptococcal meningitis	1	0	1 (2.6)
Toxoplasmosis	1	0	1 (2.6)
Lymphoma	0	1	1 (2.6)
Pulmonary embolus	1	0	1 (2.6)
Severe sepsis	0	1	1 (2.6)
Other meningitis	0	1	1 (2.6)

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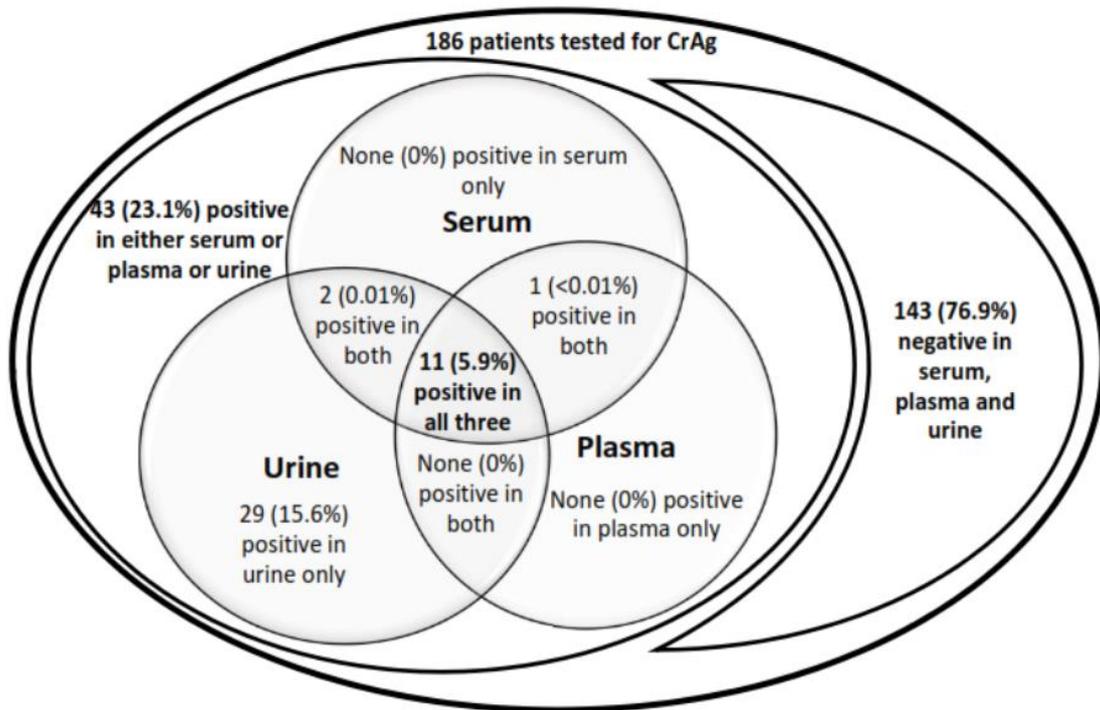


Figure 1: Venn diagram showing the prevalence of CrAg in serum, plasma and urine with the IMMY LFA test

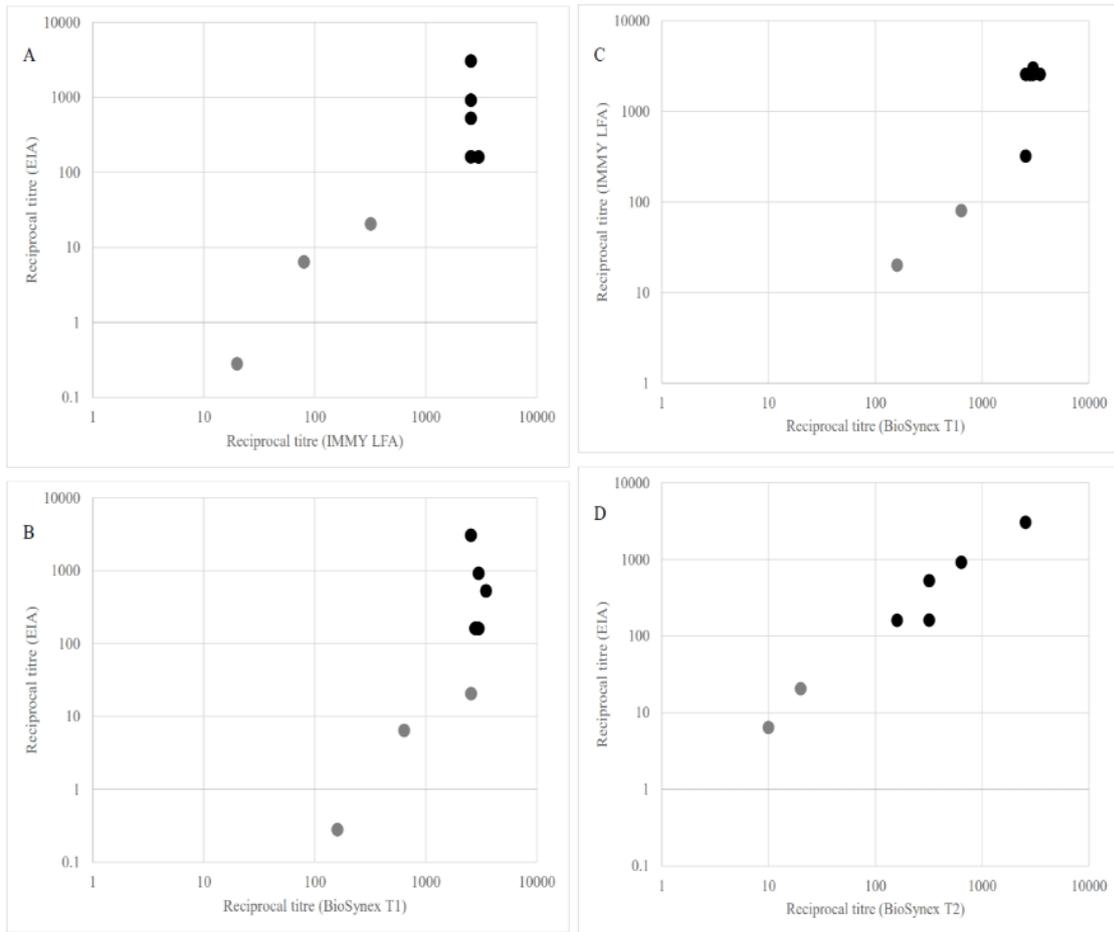


Figure 2: Scatter plots with highlighted points of agreement of CrAg test in classifying patients with elevated serum CrAg. A: EIA CrAg vs. IMMY LFA, B: EIA CrAg vs. Biosynex T1-band, C: IMMY LFA vs. Biosynex T1-band and D: EIA CrAg vs. Biosynex T2-band

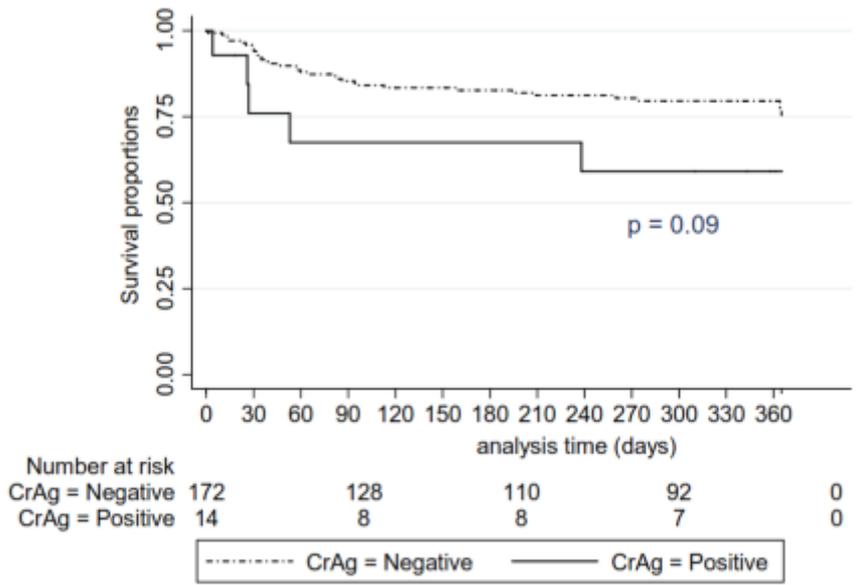


Figure 3: Kaplan-Meier survival estimates by CrAg status during first year of follow-up.

2.3. Cryptococcal antigen detection and diagnosis: systematic review

2.3.1. Summary

Background

In settings with high burden of cryptococcal meningitis (CM), late diagnosis is a major factor associated with poor outcome. With the availability of simpler cryptococcal antigen (CrAg) tests, CrAg detection as a surrogate marker of confirmed CM in clinical suspicion of CM could facilitate timely institution of induction antifungal therapy.

Method

To systematically assess the diagnostic value of CrAg detection in HIV patients with suspected CM, we searched MEDLINE, EMBASE and Web of Science for studies published from January 1981 to February 2017 in which CrAg detection in serum and/or cerebrospinal fluid (CSF) were compared to standard Indian ink staining and/or culture of CSF. Included studies were assessed for bias using the Quality assessment of Diagnostic Accuracy studies version 2 (QUADAS-2). Results were synthesised using bivariate model meta-analysis and univariate model when there was insufficient number of studies.

Results

Eight studies in which CrAg detection in 778 sera and 2483 CSF samples from 1579 participants were included. Risk of bias was low. The prevalence of confirmed CM was 46% (IQR: 42 – 68%). Point estimates of sensitivity and specificity compared to CSF culture in serum were 100% and 96% (95%CI: 87 – 99) respectively and in CSF 99% (95%CI: 93 – 99) and 99% (95%CI: 92 – 100) respectively.

Conclusion

In HIV patients with clinical suspicion of CM, serum and CSF CrAg detection are highly concordant with CSF fungal evidence of CM. CrAg detection in serum of these patients is presumptively diagnostic, requiring referral for lumbar puncture or consideration for combination antifungal therapy. CrAg detection in CSF which ideally is sterile, is diagnostic evidence of confirmed CM.

2.3.2. Draft article 3: for submission to Clinical Microbiology and Infection

BACKGROUND

Cryptococcosis, a life threatening systemic opportunistic fungal infection of which cryptococcal meningitis (CM) is the most severe, occurs in patients with defective cell mediated immunity [46, 47]. Consequent to acquired profound immune depression associated with the human immune deficiency virus (HIV) pandemic [128, 129], there has been a surge in the burden of CM, especially in low and middle income countries (LMIC) where more than 90% of CM is HIV-related, resulting in up to 15% of HIV-associated all-cause mortality [52].

Confirmatory diagnosis of CM traditionally relies on laboratory identification of rounded unicellular encapsulated yeasts by microscopy of fresh cerebrospinal fluid (CSF) obtained by lumbar puncture (LP) after Indian ink staining or culture in Sabouraud dextrose agar [70, 72]. Confirmatory diagnosis therefore requires technical expertise as well as laboratories equipped enough for the procedure, not always available in most LMIC settings with highest burden of CM. More so, patients' acceptance of LP in such settings is not always guaranteed [82, 83] thus the need for alternative reliable diagnostic approaches for timely institution of recommended treatment [72].

Cryptococcus characterized by a polysaccharide capsule known as cryptococcal antigen (CrAg), can be detectable in biological milieus days to weeks prior to appearance of symptoms [54]. Therefore, in the presence of symptoms of central nervous system (CNS) disease, CrAg detection could be a determinant surrogate marker of fungal presence for timely CM diagnosis. Within the last half century, much interest on CrAg detection has led to the availability of many commercialized CrAg tests based on antibody-antigen interactions using different principles among which: latex agglutination (LA), enzyme linked immunosorbent assays (ELISA) [130, 131], and more recently immunochromatographic lateral flow assays (LFA) [103, 126]. The earliest of these tests was in 1963 when anti-cryptococcal globulin coated latex particles were used to agglutinate CrAg in CSF and serum [132]. At the time, such a rapid, highly sensitive and specific test was revolutionary [112], resulting in its adoption as reference CrAg detection standard by many diagnostic laboratories [133, 134]. However, LA has its flaws among which false positive results (due to interference with rheumatoid factor [135, 136], syneresis fluid from agar [137], antigens some other microorganisms [138, 139], disinfectants [140]) and false negative results (CrAg clogging by unknown proteins [141], prozone effect due to excess CrAg in samples [142]). These flaws though remedied by systematic prior enzymatic denaturation of excess non-specific proteins with pronase or prior reduction of CrAg concentration by serial

dilution [141, 143, 144] are demanding in technical experience and time. ELISA CrAg detection, unlike LA requires no pre-treatment of samples, provides automated spectrophotometrically determined objective results [145] but requires trained personnel, more sophisticated laboratory infrastructure (for performing tests and reagents storage) and is not cost sustainable for single patient testing. Most recently, CrAg detection has evolved from being exclusively a laboratory technique to point of care (POC) through the development of immunochromatographic lateral flow assay (LFA) tests which are cheaper, detect all serotypes, are faster (results in ten minutes) and pose little constraints on storage (room temperature) and technical expertise [103, 126]. Irrespective of constraints, well performed LA, ELISA or LFA CrAg tests provide reliable results of high clinical importance.

In the present global context where CrAg detection tests are readily available, simpler and affordable and the prevailing difficulties in providing standardized CM diagnosis in LMIC settings, establishing the clinical utility of CrAg detection in the clinical pathway (Figure 1) of severely immune depressed HIV patients presenting with symptoms of CNS disease is crucial for the timely diagnosis and treatment of CM. We therefore performed a systematic review and meta-analysis to determine the diagnostic accuracy of CrAg detection in serum and/or CSF with commercially available tests in HIV patients clinically suspected of CM compared to CSF Indian ink staining and fungal culture [70].

METHODS

Protocol registration

The protocol of the current review was registered to the university of York centre for reviews and dissemination, PROSPERO international prospective register of systematic reviews 2017, reference CRD42017069664.

Search strategy and study selection

Comprehensive searches for published and unpublished studies in MEDLINE (via PubMed), EMBASE, and Web of Science were developed by a medical information specialist (RS). Medical subject headings (MeSH) terms and other search words including: “cryptococcal antigen”, “cryptococcal surface polysaccharide”, “cryptococcal meningitis”, “HIV”, “AIDS” as well as the names of the underlying principles of the index tests (As seen in appendix for Medline search) were used. Searches were run from 1981 (year first HIV case) through February 2017. Due to heterogeneity in reporting diagnostic test accuracy (DTA) studies and the absence of indexing consensus for such studies [146], to avoid omitting relevant studies, we

refrained from using methodological filters. To capture studies not indexed on MEDLINE, PubMed open searches with phrases of the review question were also done. We also did google scholar searches for reports that cited included articles. Conference proceedings of the annual international conference on Cryptococcus and cryptococcosis (ICCC), Conference on Retroviruses and Opportunistic Infections (CROI) and international AIDS society (IAS) conferences from 2010 onwards were also screened.

Following the electronic search, identified duplicates were removed and the resultant reference list exported to the Rayyan systematic reviews web-based application (<http://rayyan.qcri.org>) where two review authors (ET and JJBR) independently screened citations for eligibility. Discrepancies were resolved by discussion and if not resolved, a third member of the review team (JFC) was contacted for consensus. Studies published in English, French and Spanish were included and no relevant studies in other languages was found.

We included randomized trials, cross-sectional or cohort (prospective and retrospective) studies in which CrAg detection (using LA, ELISA or LFA-based principle considered the index test) was done on serum and/or CSF of HIV-patients presenting with signs and symptoms evocative of CM in whom concomitant CM diagnostic CSF analysis was done. CM ascertainment was by yeast identification after Indian ink staining and/or culture of fresh CSF, considered the reference standard diagnostic test as defined by the European Organisation for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycosis Study group (EORTC/MSG) Consensus Group [70]. In this context, patients with positive fungal culture and/or Indian ink stain in CSF were considered as having CM; those with both a negative fungal culture and negative Indian ink staining were considered as not having CM. Studies in which composite reference standards comprising the above-mentioned standards were used, data extraction and analysis considered its impact on the results. Study participants had to be aged at least 18 years, irrespective of sex or ART status (naïve or experienced). Single or multi-center studies, irrespective of the country, region or continent, and level of care (primary, secondary or tertiary) were included. Case-control studies were excluded due to high propensity to bias in diagnostic test evaluation [147].

Data extraction and quality assessment

Data was extracted on a piloted pre-specified form. Studies which evaluated more than one index test were subdivided and data for each index test extracted independently and identified with a number in brackets after the author name. Information extracted included:

- Data for constructing 2 x 2 contingency tables. Test results were binary (positive or

negative): number of true positives, false positives, true negatives and false negatives, number of indeterminate results. Though some studies did antigen titration following CrAg testing, titer levels were not considered in the review.

- Study characteristics: first author, year of publication, type of report (journal article, conference abstract), study design (cross sectional, cohort: prospective vs. retrospective), study setting (country, continent), patient inclusion and exclusion criteria;
- Participant characteristics: number of participants, age (mean or median), sex, ART status (naïve or experienced), CD4 counts (mean or median), survival history, other clinical characteristics when available;
- Index test characteristics: commercial name of the kit, test principle (LA, ELISA or LFA), types of biological samples used (serum, CSF or both), total number of samples tested, technical specifications for testing (heat inactivation and pronase pretreatment or dilutions prior to LA testing),
- Reference test characteristics: commercial name if available, underlying principle, technical specifications;
- Any other information of relevance (funding, comments on the report).

Risk of bias was assessed using the Quality Assessment of Diagnostic Accuracy Studies 2 (QUADAS-2) tool [148]. This four-domain tool was adapted to suit the review question and for each of the first three domains (patient selection, index test, and reference standard), the risk of bias as well as the applicability to the review were evaluated and classified as either “low risk”, “high risk” or “unclear” (if insufficiently reported details) and for the last domain (flow and timing), risk of bias was evaluated. In studies reporting the results for multiple index tests, each index test was assessed individually.

Statistical analysis and data synthesis

Data analysis was done with Stata 13.1 statistical package (Statacorp, Texas, USA) and Review manager (Revman) software version 5.3 [111]. Based on data from the 2 x 2 tables, for each index test, sensitivity and specificity in serum and/or CSF were estimated using Revman, reported as percentages with their 95% confidence interval (CI) and illustrated on forest plots. Bivariate random effect models were used to estimate pooled sensitivity and specificity by test principle and by sample type used. In case of insufficient number of studies, univariate random effect models and likelihood ratio tests were used to calculate summary estimates of sensitivity and specificity.

RESULTS

Results of the search

The comprehensive electronic search done on the 22nd of February 2017, identified 1966 citations (293 duplicates) of which 1639 were excluded based on titles and abstract screening mainly because of irrelevance to the review question (Figure 2). After further assessment of 34 citations, we excluded 26 studies [95, 112, 130, 141, 145, 149-169] for various reasons (Table 2).

We included 8 studies [68, 90, 131, 170-174] published between 1990 and 2015 (Table 1), carried out in 7 countries (5 in LMIC and 2 in HIC) on 1579 adult HIV patients who presented with signs and symptoms relevant to clinical suspicion of CM. The median reported age was 35.5 years (IQR: 33 – 38). The median sample size was 112 participants (IQR: 87 – 207) predominantly males at median distribution of 71% (IQR: 49.5 – 85) as reported in three studies and a median CD₄ cell/ml of 27 (IQR: 25 – 27).

Overall, these studies raised little concerns on risk of bias (Figures 3 and 4). In all 8 studies, CSF fungal culture was always part of the reference standard for the confirmatory diagnosis of CM: 6/8 (75%) used culture and Indian ink, 3/8 (37.5%) used a composite standard (CSF culture, Indian ink and CrAg tests) [68, 172, 174]. No study relied solely on Indian ink as reference standard.

The median reported prevalence of confirmed CM (based on reference standard) in these studies was 46% (Interquartile range [IQR]: 42 – 60.8).

Index tests evaluated

CrAg detection was done in CSF in 7/8 (87.5%) of included studies, in serum in 5/8 (62.5) studies and in both serum and CSF in 4/8 (50%) studies. This yielded a total of 778 sera and 2483 CSF test results.

By CrAg detection principle, 7/8 (87.5%) evaluated LA (on 560 sera and 1498 CSF), 3/8 (37.5%) evaluated LFA (on 218 sera and 985 CSF) and none of the selected studies evaluated ELISA (Table 1). In total, there were 13 diagnostic index test evaluations: 2/8 (25%) studies evaluated three tests, [68, 131], 1/8 (12.5%) evaluated two tests [172] and 5/8 (62.5%) studies, just one test [90, 170, 171, 173, 174]. In all, 6 commercial CrAg tests were evaluated, including 5 consisting in LA detection: Pastorex (Sanofi Diagnostic Pasteur, France), Cryptococcal antigen latex agglutination system (CALAS, Meridian Biosciences, USA), Latex agglutination CrAg (IMMY Diagnostics, Oklahoma, USA), Crypto-Latex agglutination (Crypto-LA,

International Biological Labs, Cranberry, NJ, USA), Cryptococcal latex agglutination (Fumouze, France) and IMMY LFA (IMMY Diagnostics, Oklahoma, USA).

Serum CrAg detection in clinical suspicion of HIV-related CM

Evaluating LA tests in 560 serum samples in 5 studies, sensitivity was 100% and specificity ranged from 95 – 100% (Figure 5). Pooled serum LA CrAg: sensitivity was 100% (95%CI: 100 – 100) and specificity of 97% (95%CI: 94 – 99).

Evaluating IMMY LFA in 218 sera in one study (Figure 5), sensitivity was 100% (95%CI: 97 – 100) and specificity was 75% (95%CI: 65 – 83).

Pooling all the studies in which CrAg detection was done in serum irrespective of test principle used, sensitivity was 100% (95%CI: 100 – 100) and specificity was 96% (95%CI: 87 – 99%).

CSF CrAg detection in clinical suspicion of HIV-related CM

Evaluating LA in 1498 CSF samples in 6 studies, sensitivity ranged from 80 – 100% and specificity from 82 to 100% (Figure 6). Pooled together, CSF LA CrAg: sensitivity was 97% (95%CI: 93 – 99) and specificity was 99% (95%CI: 92 – 100).

Evaluating IMMY LFA in 985 CSF samples in three studies (Figure 6), sensitivity ranged from 99 – 100% and specificity from 85 – 100%.

Pooling all studies in which CrAg detection was done in CSF irrespective of test principle used, sensitivity was 99% (95%CI: 93 – 99) and specificity 99% (95%CI: 92 – 100).

DISCUSSION

The main findings of this systematic review and meta-analysis show that commercially available CrAg detection tests are highly sensitive and specific in serum and CSF in predicting the presence of confirmed CM in HIV infected patients presenting with symptoms evocative of CM.

In routine practice, the utility of a test depends on the role played by test results in guiding clinical decisions that directly impact patient outcome. CM, a severe disseminated disease with cerebral involvement requires timely institution of combination antifungal therapy to avoid poor outcomes associated with late treatment institution [72, 175]. Considering the high cost of CM treatment [105] and potential adverse events associated with currently recommended induction treatment [176], deciding to treat a patient for CM, whose clinical expression is non-specific, is challenging. As such, intervening to impact patient outcome is totally dependent the test's ability to correctly classify patients with and without disease.

In most LMIC settings with high burden of CM and no facilities for CSF analysis, an algorithm combining low threshold of clinical suspicion of CM and serum CrAg detection as presumptive diagnosis could be suggested. When we pooled results of serum CrAg detection in patients with clinical suspicion of CM in the HIV context, serum CrAg positivity perfectly agreed with culture in classifying patients with CM and wrongly classified only a few without CM as having confirmed CM (specificity 96%). This means that adopting systematic serum CrAg detection in HIV patients presenting with symptoms of CNS disease, potentially identifies all patients with CM who subsequently can be referred for LP and/or considered for combination antifungal therapy. This seems plausible as a strategy in settings with low LP uptake, which ensures treatment of all patients with CM but may subjects a few patients to combination antifungal therapy albeit absence of CM. With recent evidence on the equivalence of oral combination of high dose fluconazole and flucytosine as inductive therapy for CM [177], could consider oral treatment as inductive treatment of choice for presumptively diagnosed CM. Acceptably, a few patients will be falsely treated, but false treatment is still beneficial considering the rationale for high dose fluconazole for pre-emptive therapy in asymptomatic CrAg positive patients [72]. Therefore, routine serum CrAg detection in symptomatic patients could serve as triage for those to undergo LP and those to be considered for other causes of symptoms if test is negative, thereby speeding diagnosis.

Relying on Indian ink staining of CSF and/or culture as confirmatory diagnosis of CM requires a laboratory setting, trained technicians and sustainable reagents and equipment. Moreover, Indian ink staining of CSF may be less sensitive, at least in some studies, (86% [68, 69]) requires high fungal count and CSF centrifugation in most cases for the test to be positive. Fungal culture though reliable, requires viable organisms in CSF and laboratory incubation at 30°C for at least 24 hours to ensure fungal growth, thereby delaying diagnosis. When we pooled CSF CrAg detection results of patients with clinical suspicion of CM, CSF CrAg strongly agreed with culture in classifying patients with and without CM. This high agreement and the availability of CrAg test capable of providing results within minutes, means that adopting low clinical threshold for CM suspicion in settings with high LP uptake will results in initiation of inductive antifungal combination therapy within hours of symptom onset. Therefore, CSF CrAg detection in this case, is a reliable rapid diagnostic test for CM especially as CSF ideally is sterile.

Our review and meta-analysis had some limitations. The LFA assay, a very promising point of care test with high applicability even in difficult settings was evaluated in few studies, most of which used composite reference for CM diagnosis, which could over-estimate the diagnostic

performance of the test and lead to flawed conclusions. Pooling together results of tests of different principles may undermine unreported limitations of each test. However, our findings could serve as template on which clinical intervention strategies could be developed for the timely diagnosis and treatment of CM.

Authors' conclusions

CrAg detection in serum and CSF of HIV-patients with symptoms suggestive of CM strongly correlates with culture and/or Indian ink confirmed CM. In settings without facilities for confirmatory diagnosis of CM or with low LP uptake, combination antifungal therapy should be considered for symptomatic patients testing positive for serum CrAg. CSF CrAg is a rapid and reliable diagnostic test for confirmed HIV-associated CM.

Table 1: Characteristics of included studies					
Author, year	Country	Population	Index test principle and commercial name	Reference standard for ascertainment of target condition (CM)	Comments
Nelson, M.R. 1990	United Kingdom	Consecutive sampling of HIV patients presenting fever and meningism in a hospital setting 828 patients with fever were screened for CrAg in serum and 69 with meningism considered for lumbar puncture	Latex agglutination (LA CrAg system from IMMY Diagnostics)	CM diagnosed by Nigrosin and/or Culture CSF collection was done in patients with meningism without considering results of serum CrAg testing	CrAg test done on both serum and CSF Though a large group of patients were serum CrAg tested, only those with meningism were tested with the reference standards
Calvo, B. 1991	Brazil	Consecutive sampling of 26 HIV patients with suspected CM in a hospital setting	Latex agglutination (Crypto-LA of International Biological labs)	CM diagnosed by Indian ink and/or Culture CSF was collected in all patients tested for CrAg	CrAg test done on both serum and CSF. CSF titres measured by enzyme immune-assay

Temstet, A. 1992	France	Consecutive sampling of 87 HIV patients from University hospitals with suspected CM whose sera was sent for routine CrAg screening	<p>Latex agglutination (CrAg LA of Meridian Biosciences)</p> <p>Latex agglutination (Crypto-LA of International Biological Labs)</p> <p>Latex agglutination (Pastorex of Sanofi Pasteur Diagnostics France)</p>	<p>CM diagnosed by positive culture, not done in the study but verifiably reported as such</p> <p>Testing with the index tests was done then clinical and laboratory data of patients were retrieved to compare with CM status: with or no CM</p>	CrAg detection performances of three latex agglutination tests done in serum and CSF
Asawavichienjinda, T. 1999	Thailand	Consecutive sampling of 100 HIV-infected patients suspected of central nervous system infections in a hospital setting	Latex agglutination (Pastorex of Sanofi Diagnostics Pasteur, France)	Cryptococcal meningitis diagnosed by Indian ink stain and/or culture of CSF	Serum CrAg was done with the aim of identifying a cut-off point of serum CrAg using LA for the screening and diagnosis of CM

Boulware, D.R. 2014	South Africa and Uganda	Stored samples from two cohorts 833 HIV patients suspected of CM	Latex agglutination (CrAg LA of Meridian Biosciences) Latex agglutination (IMMY Diagnostics) Lateral flow assay (IMMY Diagnostics)	Ascertainment of CM was by positive culture or a composite standard defined as negative culture and at least two other positive tests (Indian ink, CrAg latex or CrAg LFA)	Three index tests were evaluated in CSF The use of composite reference was justified as minimising bias of a single reference standard.
Kabanda, T. 2014	Uganda	Prospective cohort of 112 HIV-infected patients suspected of CM in hospital setting included consecutively in the study	Latex agglutination (CrAg LA of Meridian Biosciences) Lateral flow assay (IMMY Diagnostics)	CM diagnosed by Indian ink and/or culture and compared to composite reference CrAg testing was done before reference standard on CSF	Two index test performances evaluated on CSF There was use of composite standard defined as more than two positive tests which were not specified

Williams, D.A. 2015	Uganda	Consecutive sampling of 207 HIV patients suspected of CM in a hospital setting	Lateral flow assay (IMMY Diagnostics)	CM diagnosed by CSF culture performance of index test compared to a composite reference	Index test evaluation on serum and CSF The aim of the study was to evaluate CrAg screening by finger prick
Kammalac, N.T. 2015	Cameroon	Consecutive sampling of 146 HIV-infected patients with suspected CNS infections in a hospital setting	Latex agglutination (LA of Fumouze Diagnostics France)	CM diagnosed by Indian ink stain and/or Culture	Index test done in CSF

Table 2: Excluded studies and reasons for exclusion.		
Author, year	Country	Reason(s) for exclusion
Desmet, P. 1989	Democratic Republic of Congo	- Partial verification with reference standard
Hamilton, J.R. 1991	United States of America	- Case control design - Mixed population of HIV and non-HIV patients
Swinne, D. 1992	Rwanda	- Sera and CSF compared in the study were from two different groups of patients
Frank, U.K. 1993	United states of America	- Target condition is not CM but cryptococcal disease whose ascertainment was not clear and could as well include pulmonary cryptococcosis
Sehkon, A.S. 1993	Canada	- No comparison to our predefined reference standard
Warren, R.J. 1993	Australia	- Case control design
Tanner, D.C. 1994	United States of America	- Not a diagnostic test accuracy study - Mixed population
Kiska, D.L. 1994	United States of America	- Comparison of two latex agglutination tests to each other with another latex test as reference standard
Negroni, R. 1995	Argentina	- Partial verification with reference standard
Jaye, D.L. 1998	United States of America	- Not clear if study population was HIV-infected patients - No comparison to our predefined reference standards

Bogaerts, J. 1999	Rwanda	- Mixed population of HIV and non-HIV patients
Kantardjiev, T. 2000	Bulgaria	- Very small sample of 7 patients
Wadhwa, A. 2008	India	- Very small sample of 5 patients
Saha, D.C. 2009	India	- Case control design
Jarvis, J.N. 2011	South Africa	- Index test was performed on patients with confirmed CM
Marak, R.S.K. 2011	India	- Mixed population of HIV and non-HIV patients
Marak, R.S.K, 2012	India	- Mixed population of HIV and non-HIV patients - Reference standard not clearly stated
Ignatyeva, S.M. 2013	Russia	- Case control design
Sow, D. 2013	Senegal	- Mixed population of HIV and non-HIV patients
Escadon, P. 2013	Columbia	- Reference standard is another CrAg test - Method of ascertainment of CM not defined in the study
Wahyningsih, R. 2014	Indonesia	- Mixed population of HIV and non-HIV patients - No comparison to our predefined reference standards
Lourens, A. 2014	South Africa	- Study population of adults and children

Chaurasia, S. 2015	India	<ul style="list-style-type: none"> - Mixed population of HIV and non-HIV patients - Mixed population of adults and children
Tang, M.W. 2016	United States of America	<ul style="list-style-type: none"> - Narrative review on IMMY CrAg LFA
Dharmshale, S.N. 2016	India	<ul style="list-style-type: none"> - Mixed population of HIV and non-HIV patients - Reference standard was polymerase chain reaction (PCR) in CSF
Kumari, S. 2016	India	<ul style="list-style-type: none"> - Mixed population of HIV and non-HIV patients - No comparison to our predefined reference standard

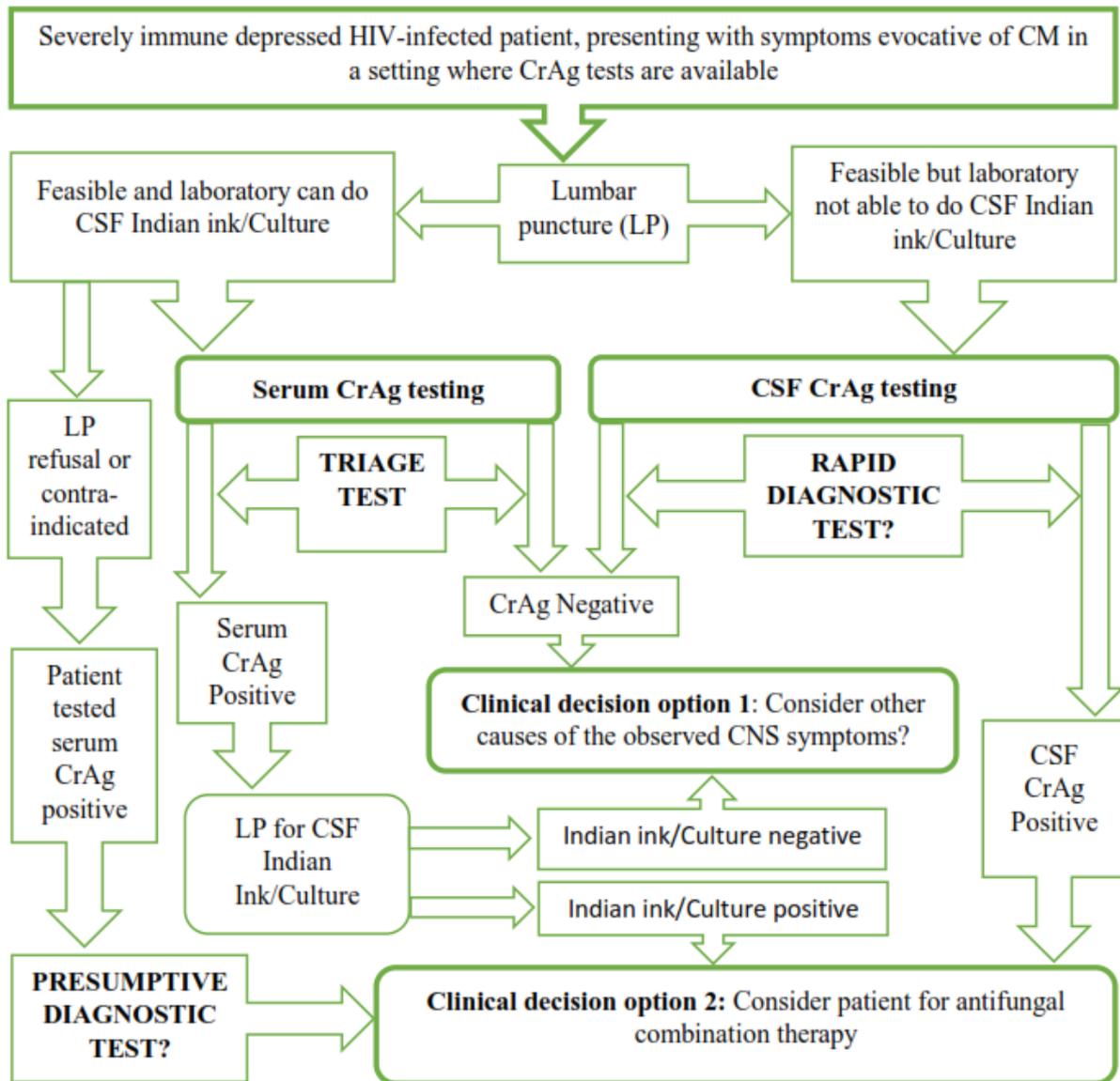


Figure 2: Patient pathway in routine settings and the role of CrAg detection on clinical decision on patient outcome

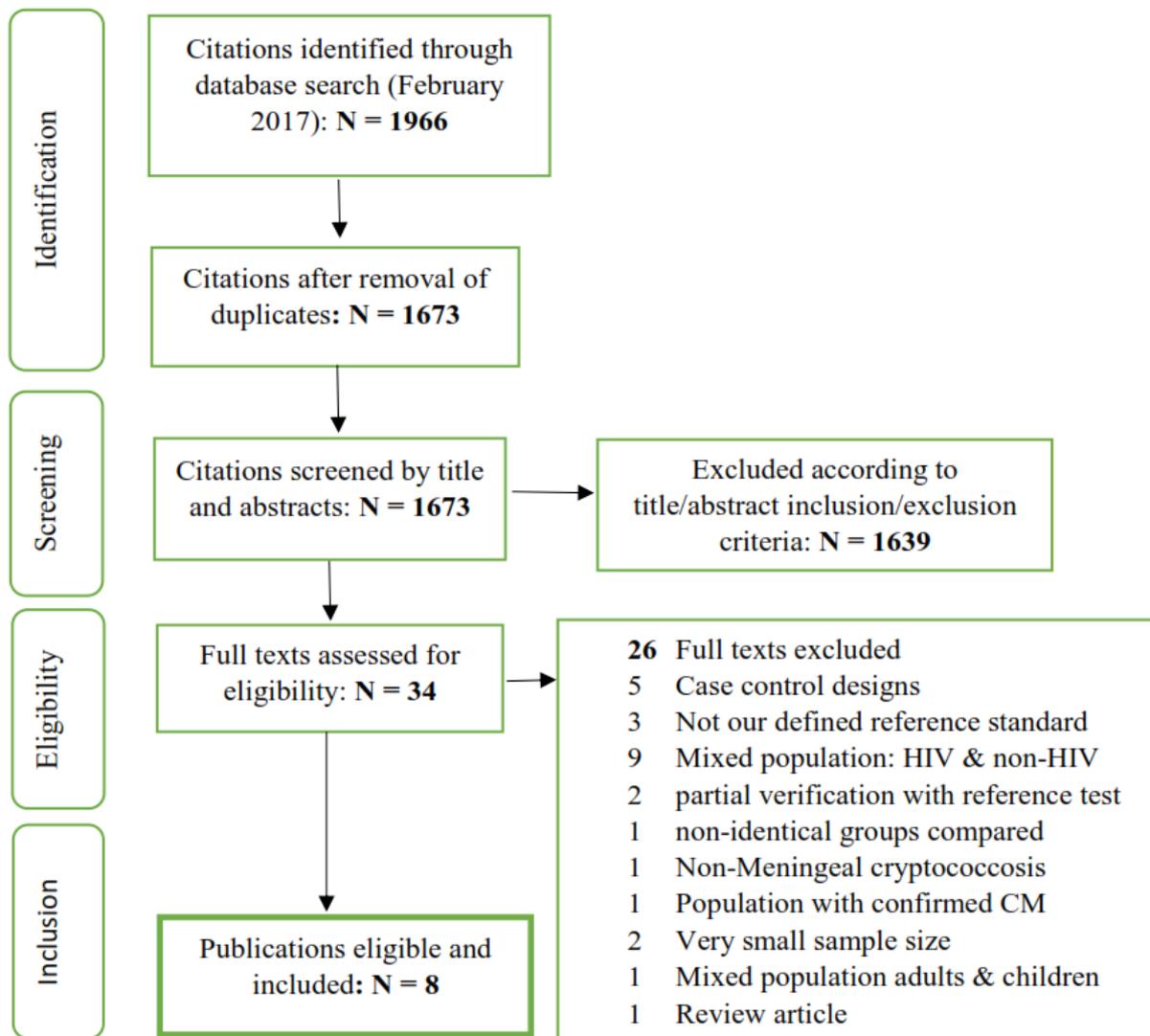


Figure 3: flow diagram of the study selection process

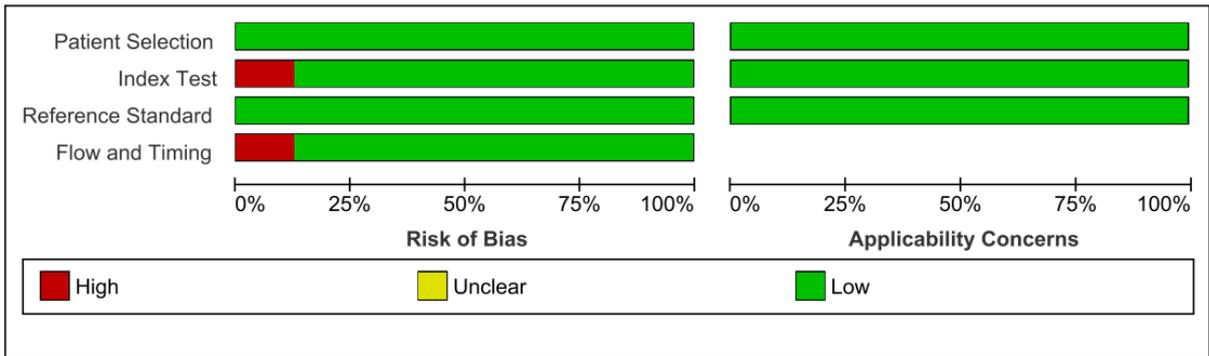


Figure 4: Risk of bias and applicability concerns graph: review authors' judgements about each domain presented as percentages across included studies (n=8)

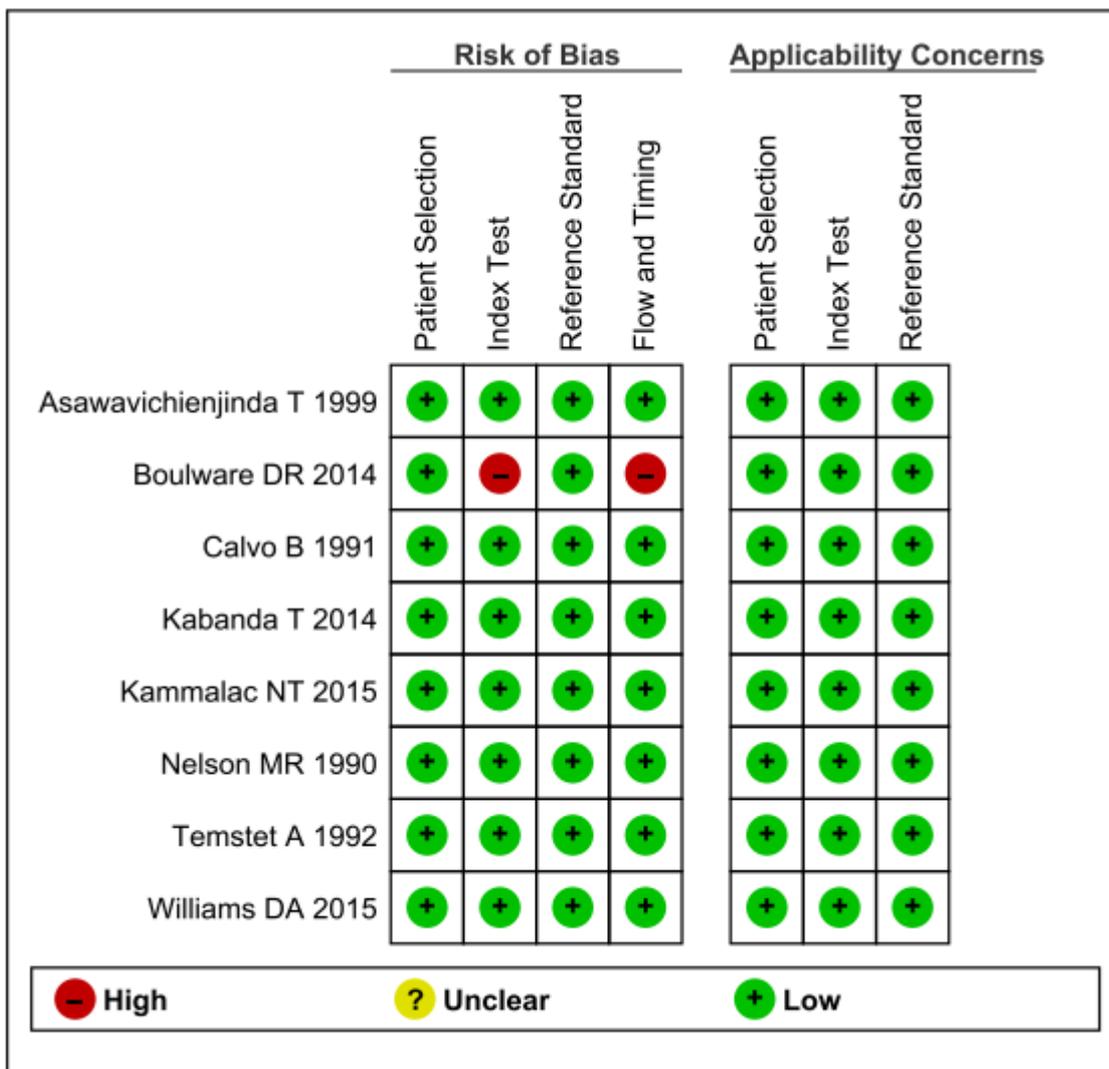
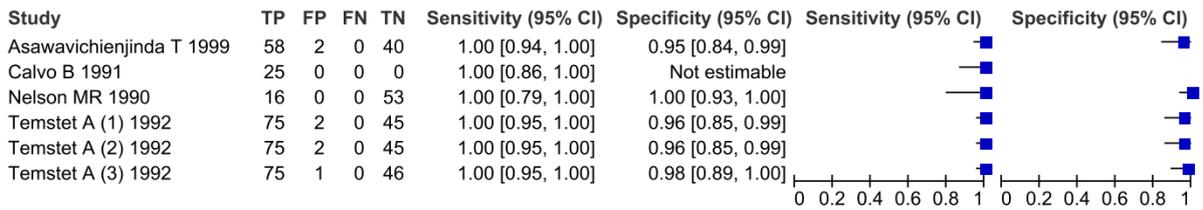


Figure 5: Risk of bias and applicability concerns summary: review authors' judgements about each domain for each included study (n=8)

LA test in serum

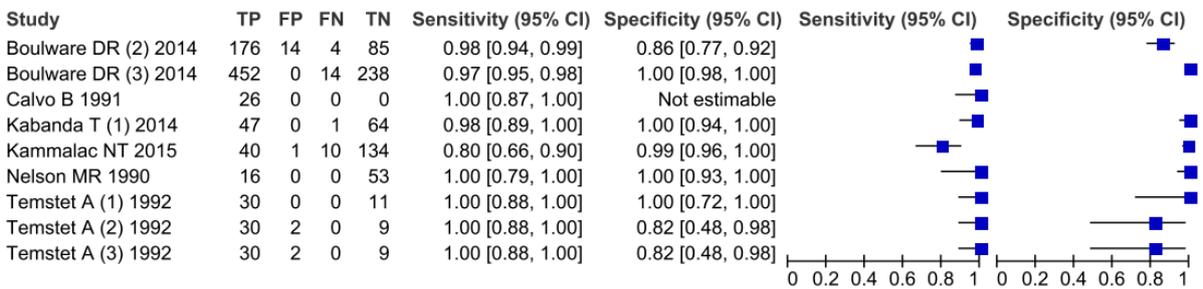


IMMY LFA in serum



Figure 6: CrAg detection in serum of severe immune depressed HIV-infected patients with clinical suspicion of CM

LA test in CSF



IMMY LFA in CSF

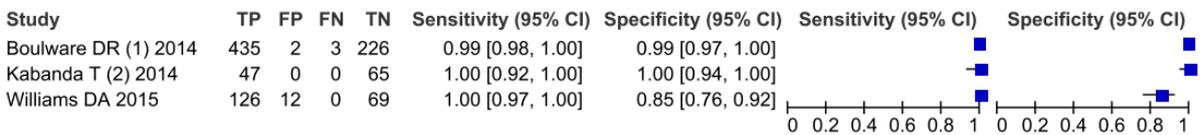


Figure 6: CrAg detection in CSF of severe immune depressed HIV-infected patients with clinical suspicion of CM

Appendix

Appendix 1: Medline search

((Meningitis, cryptococcal[MeSH] OR Antigens, Fungal[MeSH] OR Cryptococcus neoformans[MeSH] OR cryptococcosis[MeSH]) OR (cryptococcal meningitis[tw] OR cryptococcal meningoencephalitis[tw] OR toruloma*[tw] OR cerebral cryptococcosis[tw] OR cryptococc*[tw] OR Cryptococcus

AND

((Clinical Laboratory Techniques[MeSH] OR "Mass Screening"[MeSH] OR Point-of-Care Systems[MeSH] OR Latex Fixation Tests[MeSH] OR Enzyme-Linked Immunosorbent Assay[MeSH] OR Immunoassay Techniques/methods[MeSH] OR Immunologic Tests[MeSH] OR

Immunoassay[MeSH] OR Immunoassay Techniques[MeSH]) OR (screen*[tw] OR Latex agglutination[tw] OR Latex particle agglutination[tw] OR enzyme immunoassay*[tw] OR enzyme immuno-assay[tw] OR enzyme-linked immunoassay*[tw] OR ELISA*[tw] OR EIA[tw] OR Reagent

AND

((HIV Infections[MeSH] OR HIV[MeSH] OR "sexually transmitted diseases, Viral"[MeSH:NoExp]) OR hiv[tw] OR hiv-1*[tw] OR hiv-2*[tw] OR hiv1[tw] OR hiv2[tw] OR hiv infect*[tw] OR human immunodeficiency virus[tw] OR human immunodeficiency virus[tw] OR human immunodeficiency virus[tw] OR human immune-deficiency virus[tw] OR (human immun*[tw] AND deficiency virus[tw])

OR acquired immunodeficiency syndrome[tw] OR acquired immunodeficiency syndrome[tw] OR

AND

("1981"[Publication Date] : "2017"[Publication Date])

2.4. Advancing Cryptococcal meningitis treatment in Africa

2.4.1. Summary

The results of the trial as submitted to New England Journal of Medicine are under the embargo to the journal's confidentiality policy. With respect to this embargo, we presented only the abstract that was presented at the 9th International AIDS society conference

MOAX0201LB

A randomized controlled trial for the treatment of HIV-associated cryptococcal meningitis in Africa: oral fluconazole plus flucytosine or one week amphotericin-based therapy versus two weeks amphotericin-based therapy. The ACTA Trial

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Background: Cryptococcal meningitis (CM) accounts for 10–20% of HIV-related deaths and >100,000 deaths/year. Amphotericin (AmB)

plus flucytosine for 2 weeks is considered the gold standard but is unavailable in resource-limited settings where fluconazole treatment predominates.

Methods: Based on Phase II studies, we tested, against 2 weeks AmB-based treatment, 2 new strategies, which could be sustainable in Africa, and more effective than fluconazole: optimized oral therapy of high dose fluconazole plus flucytosine, and short (1 week) induction with AmB-based treatment. In the AmB arms, we compared fluconazole and flucytosine as adjunctive treatments. Between 2013 and 2016, 721 participants from 9 centres in Malawi, Zambia, Cameroon and Tanzania with first-episode CM were randomized to:

Oral (238): fluconazole (1200mg/day) plus flucytosine (100mg/kg/day) for 2 weeks.

1-week (240): AmB (1mg/kg/d), plus fluconazole (1200mg/day), or flucytosine (100mg/kg/day) (ratio 1:1), for 7 days. Days 8–14, fluconazole 1200mg/day.

2-weeks (243): AmB (1mg/kg/d) plus fluconazole (1200mg/day), or flucytosine (100mg/kg/day) (ratio 1:1), for 14 days.

After 2 weeks, all received standard fluconazole consolidation. ART was started, or restarted, at 4 weeks, and patients followed-up to 10 weeks.

Results: Only 4 participants were lost-to-follow-up. Mortality at 2 and 10 weeks for oral, 1-week and 2-weeks was 18%, 22%, 21%, and 35%, 36%, 40%, respectively. The upper 1-sided 95% CI limits for the difference in mortality comparing oral and 1-week against 2 weeks AmB-based treatment (primary endpoint) were 3.0% and 6.8%, below the pre-specified 10% non-inferiority margin. Hazard ratios (95% CI) were 0.82 (0.54–1.25) and 1.01 (0.68–1.51) at 2, and 0.83 (0.61–1.13) and 0.89 (0.66–1.21) at 10 weeks, for oral and 1-week versus 2-weeks, respectively. As adjunctive treatment with AmB, flucytosine was superior to fluconazole (HR(95% CI): 1.62 (1.19–2.20) $p = 0.002$). One week AmB plus flucytosine had the lowest 10-week mortality (24%), significantly lower than all other AmB arms (HR(95%CI): 0.56(0.35–0.91) comparing 1-week with 2-weeks AmB plus flucytosine). Side effects were more frequent with 2 weeks AmB than with 1 week AmB, or oral therapy.

Conclusions: One week AmB plus flucytosine and the oral combination provide safe, effective and sustainable induction therapy in resource-limited settings. Flucytosine should be made widely available for treatment of cryptococcosis.

Journal Information

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2.4.2. Draft article 4: In revision in the New England Journal of Medicine



Please review the Supplemental Files folder to review documents not compiled in the PDF.

Fluconazole Flucytosine Combination and Short Course Amphotericin-based Therapy for Cryptococcal Meningitis in Africa

Journal:	<i>New England Journal of Medicine</i>
Manuscript ID	Draft
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2.5. Tolerability of inductive cryptococcal meningitis treatment in Africa

2.5.1. Draft article 5: Ongoing analysis

INTRODUCTION

Within the last three decades, there has been a remarkable surge in the burden of cryptococcal meningitis (CM), an opportunistic fungal infection common in people with impaired T-cell mediated immunity [46-48]. This surge especially in low and middle-income countries (LMIC) is consequent to T-CD4 cell depletion secondary to the HIV pandemic where more than 90% of CM is HIV-related [51, 95, 99].

CM which inevitably leads to death if untreated, requires long lasting treatment over many months [102]. Present recommended standard induction treatment [102] relies on 14 days intravenous amphotericin B (AmB), a polyene macrolide [178] in combination with oral flucytosine, a nucleoside analogue derivative [74, 179]. AmB exerts a dose dependent fungicidal activity [180, 181] by binding to ergosterol, a fungal cell specific sterol, altering cell permeability, causing leakage of its components thereby resulting in cell death [178]. This AmB affinity to sterols is also demonstrated on mammalian cells where it binds to cholesterol and alters cell function, partly explaining some of the commonly observed treatment-related adverse events (AEs) [176]. These AEs of which renal damage and anaemia are the most significant, have underlying mechanism of occurrence which are still presently poorly understood [178]. Other AEs include febrile reactions, infusion site related phlebitis, hypokalaemia and hypomagnesemia. Available evidence shows that AEs tend to occur more frequently with AmB deoxycholate (AmBd), the most commonly used formulation [182, 183]. More so, severe AEs are potentially life threatening thereby limiting to the use of AmB [184] albeit its high fungicidal activity. However, these AEs are at least partially preventable, treatable and reversible [176]. As such, routine use of AmB warrants the availability of a minimum package for toxicity prevention, monitoring and management [102].

In clinical trials on CM conducted in LMIC in which AEs were systematically monitored, 33% of patients developed anaemia, 10% kidney failure and 5.6% hypokalaemia [75, 176] resulting in many premature treatment discontinuations. However, most studies reporting frequencies of AmBd-related AEs, do so by dose of AmBd used [176, 183], by formulation types [182, 183, 185] and treatment durations [180] with very few randomised comparisons with non-AmBd treated patients [180, 184, 186, 187], an approach which could provide more insight on AmBd-specific AEs.

In Africa where three quarters of global CM burden is reported [52], there exist few prospective data on the monitoring, prevention and treatment of AmBd-related AEs [75]. During the ACTA trial [93] for the initial treatment of HIV-associated CM in Africa, two thirds of patients received an AmBd-containing regimen (a third for seven and the other third for fourteen days) and one third received a non-AmBd regimen. So far, to the best of our knowledge, this is the largest study in which real-time standardised active monitoring and treatment of all trial drugs-related toxicities has been done systematically. As such, we wish to evaluate the frequency of AEs among AmBd and non-AmBd treated patients as well the effect of stringent adverse events monitoring on the incidence of AEs and AE-related outcome of patients treated for HIV-related CM in Africa.

METHODS

Study population and treatment

The study population was that of the ACTA phase III multicentre (Cameroon, Malawi, Tanzania and Zambia) trial (described elsewhere: manuscript submitted for publication) which enrolled and treated 721 patients with first episode of laboratory confirmed HIV-associated CM [102].

These 721 patients were randomised to receive either an oral combination regimen (fluconazole and flucytosine [5FC]) or intravenous (IV) infusion AmBd combination regimen (with oral fluconazole or oral 5FC) administered at 1mg/kg/day for 7 or 14 days, thus two main groups: AmBd (two thirds) and non-AmBd (a third).

All patients with conditions which could be exacerbated by the study drugs, were excluded from the trial. Among these conditions were severe neutropenia (<500 cells/ml), severe thrombocytopenia (<50 000/ml), raised serum creatinine despite rehydration (>2.5 mg/dl), or raised liver enzymes (alanine aminotransferase, ALT > 5 times upper normal value).

Monitoring and definition of AEs

On enrolment, all patients had baseline laboratory work-up and were hospitalised for a minimum duration of two weeks irrespective of duration of induction treatment. During the in-hospital stay, trial drugs related AEs were pre-empted [176], actively monitored (according to a predefined schedule of laboratory studies) and in case of occurrence, treated. Serum creatinine, blood electrolytes (mostly potassium) were checked every three days from baseline, complete blood counts (CBC) for haemoglobin levels, total neutrophils and platelets every five days and liver transaminases (alanine aminotransferase [ALAT]) every seven days. Based the results of active monitoring, AEs were defined according to Division of AIDS (DAIDS) table

for grading the severity of adults and paediatrics adverse events [188]. This table classifies AEs into four grades: grade 1 as mild, grade 2 as moderate, grade 3 as severe and grade 4 as very severe (potentially life threatening). Grade 3 and 4 AEs were regarded as severe adverse events (SAEs).

Pre-emptive and curative treatment of AEs

Nephrotoxicity was pre-empted by preclusion of all potentially nephrotoxic medications, systematic pre-hydration prior to AmBd infusions with 1L of saline 0.9% as well as stringent fluid monitoring and optimal hydration tailored to suit individual patient needs. Prevention of hypokalaemia was by systematic infusion supplementation with 20 mmol/L of potassium chloride (KCl) during pre-AmBd pre-hydration as well as oral supplementation with slow release potassium capsules administered twice daily. Magnesium glycerophosphate tablets were systematically administered to prevent hypomagnesaemia. To prevent chills and phlebitis, AmB was administered as a single infusion of daily dose in 1L of Glucose 5% solution for 4 hours with slower infusion lasting up to 6 hours and/or administration of antipyretics in case of chills and fever.

Patients who developed severe anaemia defined as haemoglobin <6.5 g/dl were transfused. Those in whom creatinine rose above the upper normal limit but <2.5 mg/dL, were further hydrated. Serum creatinine was then checked prior to the following daily dose of AmBd and if >2.5mg/dL, the dose was withheld, hydration maintained and continuous creatinine monitoring. In case of improvement, defined as decreased serum creatinine below 2.5 mg/dl, AmBd was reintroduced. However, when two consecutively controlled creatinine levels were persistently above 2.5mg/dL, daily doses of AmBd were halted and treatment switched to high dose oral fluconazole. Patients who developed hypokalaemia despite pre-AmB IV infusion and oral pre-emption, had their oral dose of KCl increased to trice daily of slow release potassium. Local infusion site erythema was managed by changing infusion site, alcohol dressing and in case of local signs of infection, treatment was with systemic anti-staphylococcal antibiotics (beta lactams).

Statistical analysis

Data was analysed with STATA 13.0 statistical package (Statacorp, Texas, USA). Categorical variables were expressed as percentages of the study population and compared using Pearson chi-Square test or Fisher's exact test as required. Continuous variables were presented as means (and standard deviation: SD) or medians (interquartile range: IQR) as required and compared using Student's t test or Wilcoxon test respectively.

The main outcomes of interest were anaemia, neutropenia, thrombocytopenia, rise in serum creatinine, hypokalaemia and rise in serum ALAT levels, graded SAEs according to DAIDs classification [188]. Incidence of SAEs was defined as the total number of recorded events per outcome of interest during the observation time at risk from baseline and reported per 100 person-days of follow up.

Marginal extension of the Cox proportional hazard model for multiple failure-time was used to analyse hazards of recurrent SAEs during the study period. As such, independent observation from baseline was assumed for each outcome, conditioned on covariates and ordered by study day. Results were expressed as hazard ratios (HR) with their 95% confidence intervals (CI) and covariates with p-values <0.1 in the univariate analysis, were included in the multivariate analysis and reported as adjusted hazard ratios (AHR) with 95%CI. Evidence of association was considered for a p-value <0.05.

RESULTS

Study population at baseline

A total of 721 participants were included in the trial, 58% (418/721) of whom were men. Mean age was 38.2 years (SD: 9.2) and median CD4 count was 26 cells/ml (IQR: 10 – 63). Median haemoglobin level and platelets count were respectively 10.8 g/dl (IQR: 9.5 – 12.2) and 219 000 /ml (IQR: 156 000 – 297 000), higher in men than women (p<0.001). Median absolute neutrophil count was 2500 cells/ml (IQR: 1600 – 3780), with no difference between men and women (p=0.22). Median serum creatinine was 0.7 mg/L (IQR: 0.6 – 0.9) and kalaemia was 3.7 mEq/L (IQR: 3.4 – 4.2) and both were higher in men than women (p<0.001). Previous antiretroviral therapy (ART) exposure was reported by 57.3% (412/721). Study participants were randomised into three main combination antifungal treatment arms: 33% (238/721) oral (fluconazole plus flucytosine), 33.3% (240/721) one-week combination with AmBd and 33.7% (243/721) two weeks combination with AmBd. There were no significant differences in the baseline characteristics among the treatment arms (Table 1).

Incidence of severe adverse events

During the two-week inductive treatment period, a total of 4535 observations were made on 721 patients for 60367 days-at-risk. Within this period, severe anaemia was the most common SAE (Table 2) which occurred at an incidence rate of 1 per 100 person-day of observation. Incidence of other SAEs including neutropenia: 0.6 per 100 person-day, thrombocytopenia: 0.18 per 100 person-day, rise in serum creatinine: 0.2 per 100 person-day, hypokalaemia: 0.2

per 100 person-day and rise in ALAT: 0.1 per 100 person-day. Death as an SAE occurred at incidence rate of 21 per 100 person-day of observation

Factors associated with incidence of SAEs

Hazard ratio for each of the adverse events over time, then control for factors among which regimen (AmB/no AmB), duration of AmB (7/14), combination therapy (fluco/5FC) age, sex, ART status,

Table 1: Base line characteristics of study participants by treatment arm.

Baseline Characteristics	Oral arm (n = 238)	One-week AmB (n = 240)	Two-weeks AmB (n = 243)
Female sex, no. (%)	112 (47.1)	92 (38.3)	99 (40.7)
ART exposure, no. (%)	137 (57.8)	131 (54.6)	144 (59.5)
CD4 cells/ml, median (IQR)	25 (10 – 63)	28 (12 – 65)	24 (10 – 63)
Haemoglobin g/dL, median (IQR)	10.6 (9.1 – 12.1)	10.9 (9.9 – 12.3)	10.8 (9.5 – 12.4)
Neutrophil x 10³cells/ml, median (IQR)	2.5 (1.7 – 3.7)	2.4 (1.5 – 3.5)	2.5 (1.6 – 3.9)
Platelets x 10³/ml, median (IQR)	223 (162 – 292)	210 (152 – 299)	226 (156 – 301)
Creatinine mg/L, median (IQR)	0.7 (0.6 – 0.9)	0.8 (0.6 – 0.8)	0.7 (0.6 – 0.9)
Potassium, mEq/L, median (IQR)	3.7 (3.3 – 4.1)	3.8 (3.4 – 4.2)	3.7 (3.4 – 4.1)
ALAT in IU/L, median (IQR)	21 (14 – 33)	22 (15 – 35)	22.5 (15 – 35)

Table 2: Overall frequency of severe adverse events (SAE) and by treatment arm

	Overall, n (%)	Oral arm n (%)	One-week AmB, n (%)	Two-weeks AmB, n (%)
Severe adverse event, (total observations)				
Anaemia, (N = 2618)	338 (12.9)	58 (2.2)	102 (3.9)	178 (6.8)
*Neutropenia (N = 2540)	206 (8.1)	75 (3.0)	74 (2.9)	57 (2.2)
Thrombocytopenia (N = 2617)	64 (2.4)	18 (0.7)	30 (1.1)	16 (0.6)
Rise in creatinine (N = 4242)	112 (2.6)	35 (0.8)	24 (0.6)	53 (1.2)
Hypokalaemia (N = 4242)	103 (2.4)	21 (0.5)	38 (0.9)	44 (1.0)
Rise in ALAT (N = 1950)	32 (1.6)	8 (0.4)	10 (0.5)	14 (0.7)

*78 observations missing for 11 patients

Table 3: Factors associated with haematological SAEs

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P-value	AHR (95% CI)	P-value
Anaemia				
Male sex	0.6 (0.4 – 0.8)	<0.001	0.6 (0.4 – 0.8)	<0.001
AmBd regimen	2.4 (1.6 – 3.7)	<0.001	2.1 (1.3 – 3.3)	<0.001
AmBd >1week	1.5 (1.1 – 2.1)	0.01	1.5 (1.1 – 2.0)	0.01
5FC regimen	0.5 (0.4 – 0.7)	0.001	0.7 (0.5 – 0.9)	0.03
5FC >1 week	1.1 (0.7 – 1.7)	0.6	/	/
Fluconazole regimen	0.9 (0.7 – 1.3)	0.7	/	/
Fluconazole > 1 week	1.1 (0.7 – 1.7)	0.6	/	/
Neutropenia				
Male sex	1.2 (0.8 – 1.7)	0.5	/	/
AmBd regimen	0.9 (0.6 – 1.3)	0.6	/	/
AmBd >1week	0.7 (0.5 – 1.2)	0.2	/	/
5FC regimen	1.4 (0.9 – 2.2)	0.1	1.4 (0.9 – 2.2)	0.1
5FC >1 week	0.7 (0.5 – 1.1)	0.2	/	/
Fluconazole regimen	0.8 (0.6 – 1.2)	0.3	/	/
Fluconazole > 1 week	0.6 (0.4 – 1.0)	0.04	0.7 (0.4 – 1.1)	0.1
Thrombocytopenia				
Male sex	2.9 (1.4 – 6.1)	0.004	3.2 (1.3 – 8.4)	0.02
AmBd regimen	1.2 (0.5 – 2.7)	0.7	/	/
AmBd >1week	0.5 (0.2 – 1.1)	0.08	0.5 (0.2 – 1.1)	0.09
5FC regimen	0.8 (0.4 – 1.6)	0.5	/	/
5FC >1 week	0.4 (0.2 – 1.0)	0.04	0.5 (0.2 – 1.0)	0.06
Fluconazole regimen	1.1 (0.5 – 2.2)	0.9	/	/
Fluconazole > 1 week	0.6 (0.3 – 1.2)	0.2	/	/

Table 4: Factors associated with hypokalaemia, rise in Creatinine and rise in ALAT

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P-value	AHR (95% CI)	P-value
Hypokalaemia				
Male sex	0.4 (0.3 – 0.7)	<0.001	0.4 (0.3 – 0.7)	<0.001
AmBd regimen	2.0 (1.2 – 3.5)	0.01	2.1 (1.2 – 3.7)	0.01
AmBd >1week	1.2 (0.7 – 2.3)	0.5	/	/
5FC regimen	0.8 (0.5 – 1.4)	0.4	/	/
5FC >1 week	0.7 (0.4 – 1.2)	0.4	/	/
Fluconazole regimen	0.7 (0.4 – 1.2)	0.2	/	/
Fluconazole > 1 week	0.8 (0.4 – 1.6)	0.5	/	/
Rise in Creatinine				
Male sex	1.2 (0.7 – 2.2)	0.5	/	/
AmBd regimen	1.1 (0.6 – 2.0)	0.7	/	/
AmBd >1week	1.5 (0.8 – 3.0)	0.2	/	/
5FC regimen	0.6 (0.3 – 1.1)	0.1	/	/
5FC >1 week	0.9 (0.4 – 1.8)	0.7	/	/
Fluconazole regimen	1.5 (0.8 – 2.9)	0.2	/	/
Fluconazole > 1 week	0.6 (0.3 – 1.3)	0.2	/	/
Rise in liver enzyme (ALAT)				
Male sex	2.9 (1.1 – 8.0)	0.04	/	/
AmBd regimen	1.5 (0.6 – 3.5)	0.4	/	/
AmBd >1week	1.2 (0.5 – 3.0)	0.6	/	/
5FC regimen	0.7 (0.3 – 1.5)	0.3	/	/
5FC >1 week	0.7 (0.3 – 1.9)	0.5	/	/
Fluconazole regimen	1.0 (0.5 – 2.2)	0.9	/	/
Fluconazole > 1 week	0.6 (0.2 – 1.7)	0.3	/	/

Table 5: Incidence of SAEs and their effect on all-cause mortality during inductive phase of CM treatment

Severe adverse events	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P-value	AHR (95% CI)	P-value
Anaemia	1.6 (1.2 – 2.0)	<0.001	1.5 (1.2 – 1.9)	0.02
Rise in creatinine	2.6 (2.0 – 3.4)	<0.001	2.4 (1.8 – 3.2)	<0.001
Neutropenia	1.0 (0.7 – 1.5)	0.8	N/A	/
Thrombocytopenia	1.5 (0.9 – 2.6)	0.1	N/A	/
Hypokalaemia	1.3 (0.9 – 2.0)	0.2	N/A	/
Rise in ALAT	0.5 (0.2 – 1.3)	0.2	N/A	/

2.6. Cryptococcal meningitis: outcome beyond ten weeks of initial treatment

2.6.1. Draft article 6: Published in Clinical Infectious Diseases

Long term mortality and disability in Cryptococcal Meningitis: a systematic literature review.

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Summary: One year after induction treatment, cryptococcal meningitis mortality reaches almost 80% in HIV-infected cohorts and more than 70% of non-HIV and HIV-infected survivors live with impairments. Early therapeutic intervention and early management of disability could significantly improve prognosis.

Running title: Long term prognosis of cryptococcal meningitis

Abstract :

Cryptococcal meningitis (CM) is the primary cause of meningitis in HIV-infected adults and an emerging disease in HIV-seronegative individuals. No literature review has studied the long-term outcome of CM. We performed a systematic review on the long-term (≥ 3 months) impact of CM (*C. neoformans* and *C. gattii*) on mortality and disability in HIV-infected and non-HIV-infected adults. Though the quality of current evidence is limited, the long-term impact of CM on survival and disability appears to be high. One-year mortality ranged from 13% in an Australian non-HIV *C. gattii* infected cohort to 78% in a Malawian HIV-infected cohort treated with fluconazole monotherapy. One-year impairment proportions among survivors ranged from 19% in an Australian *C. gattii* cohort to more than 70% in a Taiwanese non-HIV and HIV-infected cohorts. Ongoing early therapeutic interventions, early detection of impairments and access to rehabilitation services may significantly improve patients' survival and quality of life.

Keywords: Cryptococcal Meningitis, Long-Term Outcome, Mortality, Disability, Risk Factors

INTRODUCTION

Cryptococcal meningitis (CM) is a leading cause of meningitis in many low and middle income countries (LMICs) where it accounts for 15-20% of all HIV-related mortality[1]. Despite the expansion of antiretroviral therapy (ART) programmes, rates of CM remain high as many HIV-infected persons start ART late and face difficulties maintaining effective treatment[2]. Moreover, sustainable access to the current reference induction treatment, flucytosine (5-FC) and amphotericin B (AmB), is a major challenge in these settings. Fluconazole monotherapy is the only alternative but is associated with higher short-term mortality, even at high dosages[3]. Consequently, CM mortality remains high in LMICs ranging from 19% to 96%[4,5] at 10-12 weeks. In contrast, it has been estimated to range from 9% to 15% in Western Europe and North America[6,7].

Additionally, a growing number of CM-associated deaths occur in non-HIV patients in high-income countries (HIC)[8]. CM caused by *C. neoformans*, is increasingly observed among patients with non-HIV immunosuppression[8]. In parallel, *C. gattii* CM, endemic in Australia, appeared recently as an outbreak in North America, mainly among patients without apparent immunosuppression (up to 72% of cases). It appears that non HIV-associated CM (non-HIV-CM) mortality outcomes are no better than HIV-associated CM in similar settings [8].

Beyond mortality, CM survivors may experience different long-term (≥ 3 months after CM diagnosis) neurological and sensorial impairment resulting into disability and poor quality of life[6,9].

However, data on the long-term outcome of CM remain scarce [10,11]. Indeed, while short-term mortality of HIV-CM has been well reviewed [7,8,12,13], literature reviews including longer-term overall cryptococcosis mortality are limited[8,13] and none have reported long-term CM-related neuro-sensorial impairment and disability.

To address this gap, we exhaustively reviewed published data on the long-term mortality, impairment and disability following CM caused by *C. neoformans* or *C. gattii* occurring in either immunodeficient or immunocompetent adults. The following questions were addressed: what are the proportions/rates and predictive factors for mortality occurring ≥ 3 months after CM diagnosis or treatment induction? What are the nature, frequency and predictive factors of CM sequelae/impairment, disability and decreased quality of life occurring ≥ 3 months after CM treatment induction? Finally, are there differences in the ≥ 3 month prognosis between HIV and non-HIV infected patients?

METHODS

This systematic review was conducted in line with the PRISMA Statement[14].

Search strategy and study selection

PubMed/Medline, Web of Science, Cochrane Library, Embase, Global Health, LILACS and WHO online libraries were searched for studies published in English, French and Spanish between Jan 1st, 2005 and June 30th, 2015 (Figure 1). The search combined two groups of words including synonyms and MeSH terms of (group 1) “Cryptococcal Meningitis” and (group 2) “Outcome and risk factors” (see Supplementary Table 1).

All types of study design were considered for eligibility. Studies were included if they fulfilled the following criteria: 1) reporting the mortality rate/proportion and/or the proportion of sequelae/impairments/disability occurring ≥ 3 months after CM diagnosis or after induction treatment, 2) participants ≥ 18 years-old, 3) with documented first episode of CM diagnosed by positive India Ink and/or positive Cryptococcal Antigen and/or positive cerebrospinal fluid (CSF) culture (*C. neoformans* or *C. gattii*). Both immunodeficient and apparently immunocompetent patients were considered.

Studies on cryptococcomas without CM and studies where CM data could not be individualized from other causes of meningitis were excluded. Additional studies were manually searched for from reference lists of all identified articles.

Quality assessment

The quality of the selected studies was assessed at outcome level[14] using the Critical Appraisal Skills Programme (CASP) tools[15]. Domains assessed were: (1) validity: appropriate design, appropriate sampling methods, risk of bias and confounding; (2) importance: effect size, power, precision of the study; and (3) comparability/generalizability. Studies that fulfilled more than 70% of criteria were considered of good quality and those that fulfilled less than 50% of CASP criteria were excluded for providing weak evidence.

Data extraction and synthesis

For each study included, the following data were extracted: (1) study characteristics (setting, design, sample size, statistical method used, potential bias and confounding), (2) participants characteristics (eligibility criteria, age, sex, immunological, mental and ART status at diagnosis, ART timing of initiation), (3) Cryptococcus species, (4) outcome (mortality proportion/rate, impairments/disability proportions and quality of life) stratified by outcome timing (6, 12, >12 months after diagnosis) and (5) predictive factors (age, immunological status, viral load, CSF characteristics, clinical presentation, mental and ART status at diagnosis, antifungal and ART treatment received, adjunctive therapy, setting, cryptococcus species, Immune Reconstitution Inflammatory Syndrome (IRIS) occurrence, opportunistic co-infections/affections).

Quality assessment of included studies showed high heterogeneity; therefore we decided to undertake a qualitative synthesis rather than a meta-analysis, which included describing the studies, their results and

limitations without pooling estimates. Further synthesis of the results was done with predefined sub-groups by the patients' immunological status (HIV, non-HIV).

RESULTS

Study characteristics

Of the 6035 records identified during the study period, 177 were selected for full-text review, and 25 articles (24 studies: four randomized clinical trials (RCT), eleven prospective cohorts and nine retrospective cohorts) were eventually retained (Figure 1). Only one of the studies[16] was included although not fulfilling the inclusion criteria, because it provided key results on disability.

Twenty-one studies provided information on mortality outcomes, six on impairments, two on disability and none on quality of life. All continents were represented with fifteen studies from LMIC and nine from HIC.

Only six (25%) studies fulfilled at least 70% of quality criteria: two RCT and four cohorts (Table 1).

Long-term mortality and its predictive factors

In HIV-CM, seventeen articles provided information on CM-mortality (five from HIC and twelve from LMIC), and ten examined risk factors (Table 2). Five (28%) articles fulfilled at least 70% of quality criteria. Available data suggest that the high mortality rate of the first ten weeks of treatment continues to rise slowly to level off after six months of treatment [16–19]. However, evidence was weak with few studies and difference in CM management across studies. Two studies using survival analysis showed that when using both AmB-based

combination therapy and appropriately timed ART, the survival curve strongly flattens after 3 months[20,21]. There was important variation in the one-year mortality according to both setting and period: in HIC, mortality was around 50% in the pre-ART period[22] and 20% in the late ART period[22–24], while it ranged from 39.5% to 78% in LMIC[17–21,25–27]. The one-year mortality also varied according to the induction treatment received with fluconazole performing worse than AmB monotherapies, which, in turn, performed worse than AmB-based combined therapy (Figure 2 and Table 3). Nevertheless, even with the latter, the one-year mortality rate was around 40%[19,20] in LMIC and around 20%[22–24] in HIC. Other protective factors included the use of ART as shown in France[22] and Denmark[24], and, in ART-naïve patients, delaying the introduction of ART to 5 to 10 weeks after induction therapy compared to an introduction between 3 days to 2 weeks[20,28]. In LMIC, the one-year mortality observed in patients receiving aggressive Intracranial Pressure (ICP) management was lower compared to those who did not (40%[19,20] versus 59%[18], respectively).

In HIV-CM, the main independent risk factors for long-term mortality were altered neurological status[25,27], low CD4 level[23], high CSF fungal/CrAg burden[16,25], and older age[24] at diagnosis. Lastly, the evidence on an association between IRIS and higher long-term mortality is still unclear [20,29,30] with only one study of the three that addressed this question showing a significant association[29]. This may result from the small study sizes or from the IRIS definition adopted which may miss early IRIS leading to an ascertainment bias, as suggested by Boulware *et al* (2014)[20].

In non-HIV-CM, evidence on long-term mortality is weaker as only six articles (none from LMIC) were identified and none fulfilled at least 70% of quality criteria (Table 2).

One-year mortality ranged from 13.7% in an Australian cohort infected by *C. gattii* with only 28% of patients with underlying conditions [11,31] to 42.3% in a Taiwanese study with all patients having underlying conditions[23]. Non-HIV-CM mortality may continue to rise after three months, as suggested by data from Liao *et al.* [23].

The main independent risk factors for one-year mortality in non-HIV patients were delayed diagnosis[32], age>60 years[32], altered initial neurological status[23,32], high CSF CrAg[23] and non-AmB-based compared to AmB-based induction therapy[32] (Table 4). In patients with *C. gattii* CM, only high CSF CrAg was found to be independently associated with one-year mortality[11].

Long-term neuro-sensorial impairments and disability and their predictive factors

Seven studies provided evidence on neuro-sensorial impairment and disability (three for HIV-CM and four for non-HIV-CM) and only one fulfilled at least 70% of quality criteria[33] (supplementary Table 2).

In HIV-CM, up to 69.2% of survivors from a Taiwanese cohort had neuro-sensorial sequelae[23], mainly residual headache (38%), motor deficit (15%) and vertigo (15%), one year after diagnosis. In a Ugandan study, cognitive function remained impaired in 41% of survivors, although the contribution of HIV encephalitis should also be questioned [33].

Only two studies assessed the long-term disability related to HIV-CM [16,33] and none used the WHO International Classification of Functioning definition[9]. In the clinical trial of Day *et al.*, 40% of the survivors reported having some form of “disability” at six months [16] and in the cohort study reported by Carlson *et al.* 11% of the survivors declared themselves unable to work at one year [33].

Few predictive factors of long-term impairments and disability were found. Carlson *et al.* demonstrated that the risk of 12-month impaired cognition was increased in HIV-CM patients with lower CD4 level at induction. And paradoxically, persons with sterile CSF cultures after 14 days of AmB therapy had worse neurocognitive outcomes than those still culture-positive[33]. At six months, Day *et al.* demonstrated that patients treated with AmB+5-FC were half as likely to report having a “disability” than patients treated with AmB monotherapy[16].

In non-HIV-CM, findings were heterogeneous. In a cohort of patients infected with *C. gattii* without underlying conditions, the rates of neuro-sensorial sequelae among one-year survivors were between 19% and 24% [11,34]. This proportion reached 73.3% in a cohort of patients with underlying conditions (*Cryptococcus* species not known)[23]. The main impairments were vertigo (13% to 24%), visual loss (13% to 23%), hearing impairment (6% to 17%), and motor deficit (3% to 16%).

MRI sylvian fissure enhancement and CSF CrAg titres >1/256 at induction were, respectively, independently associated with hearing loss in any non-HIV-CM,[35] and neurological sequelae in *C. gattii* cohorts [11].

Comparison between HIV and non-HIV-CM

The three studies comparing long-term mortality between HIV and non-HIV-CM[23,36,37] gave contradictory results. Nevertheless, the studies’ heterogeneity in terms of proportions of ART-naïve patients for HIV-CM and in terms of proportions of patients with underlying conditions for non-HIV-CM prevents any valid comparisons.

DISCUSSION

Long-term impact of CM on mortality, impairments and disability

To our knowledge, this is the first literature review focusing on long-term outcomes of CM, including mortality, neuro-sensorial impairment and disability. Our review shows that the mortality can reach rates up to 78% in HIV-CM[27] and 42% in non-HIV-CM[23] at one year. In addition, regardless of the species studied (*C. neoformans*, *C. gattii*), we found supportive evidence for an important long-term burden of CM on impairments and disability with proportions reaching up to 70% in both HIV-infected and HIV-non-infected survivors[23]. These findings demonstrate that the long-term prognosis of CM in adults may be at least as poor as that associated with other aetiologies of encephalitis in France (33% with impairment at 3 years)[38] or, that associated with tuberculosis meningitis (TBM) (20% to 60% of mortality and 20% to 50% of impairments among survivors)[39,40].

Prevention of risk factors of long-term adverse CM outcomes

Evidence regarding risk factors for long-term outcomes was limited showing an important gap in knowledge. Each of the risk factors for long-term outcomes identified were found to be predictive in only 1 to 3 studies. Some studies did not adjust for confounders (ART[22] and IRIS[20,29] in HIV-CM patients, underlying conditions[11,32] and high CSF antigen titers[35] in non-HIV-CM patients). Some important potential predictive factors have not been studied (e.g. *Cryptococcus* species, phenotypes and genotypes, ICP management) and discordant results regarding the effect of IRIS in HIV-CM or of underlying conditions in non-

HIV-CM were reported. Moreover, definitions of some risk factors varied according to studies (abnormal neurology, fungal burden assessment tools and threshold, underlying pathologies) limiting the potential for comparison. Therefore, future research should systematically assess the long-term impact of all these risk factors using standardized and validated definitions and tools.

Nevertheless, some key actions can be identified to prevent long-term mortality, impairment and disability.

In HIV-CM, some of the well-known short-term mortality risk factors (baseline altered neurology, high fungal burden or immunosuppression)[21] seem to be also predictive of long-term adverse outcomes. Their identification could orient clinicians in providing closer clinical follow-up. More importantly, our review identified some evidence for a protective effect of AmB-based combined antifungal regimens for long-term mortality and AmB+5FC therapy on long-term disability. In addition, it has to be noted that only 1/7[33] of the cohorts assessing impairments and disability in this review provided an AmB-based combined therapy to all patients. Moreover, while ART does not appear to influence short-term HIV-cryptococcosis mortality[41], it plays a key role in reducing long-term mortality[20,22,24,26,28,42]. As in TBM[43], if introduced appropriately (after 4-5 weeks), ART might protect against other opportunistic infections[20]. Therefore, in order to prevent not only short-term but also longer-term adverse outcomes, there is an urgent need to scale up access and coverage of ART, AmB and especially 5-FC therapy, usually not available in LMICs [44]. Additionally, future research on antifungal therapy, as well as adjuvant therapy (especially IRIS and ICP management), should assess the effect on long-term outcomes, including disability.

In non-HIV-CM, the limited number of studies along with their heterogeneity preclude firm conclusion on long-term mortality risk factors. In *C.gattii* cohorts, our results suggest that underlying conditions and altered mental status at induction might be poor long-term prognostic factors[11,34]. But, these univariable associations might be confounded by other factors. For instance, experimental studies have shown that a

difference in virulence of the *C.gattii* genotype infecting Australian patients (mainly VG1)[45] compared to the one of Canadian patients (mainly VGII A)[46] might be another explanation for the difference in mortality. In general, the identified risk factors of non-HIV-CM long-term mortality advocate for early CM diagnosis in people >60 years old with underlying conditions. Nevertheless, management guidelines are based on results from trials involving patients with HIV-CM that may not apply to those with non-HIV-CM. Further multi-centric cohorts/trials, as already conducted for Solid Organ Transplant patients[47], should therefore be proposed, assessing further these potential predisposing factors and looking for the best therapeutic choice.

Poor attention to impairment and disability

Despite the fact that many studies were initially identified, few had quality data on long-term outcomes and only seven assessed long-term impairments and disability. Only three studies assessed impairments/disability and their predictive factors as a primary outcome and five did not describe the method of outcome measurement [11,23,31,34,36]. None used the WHO definition of disability[9], nor assessed quality of life. Similarly, reviews of long-term outcomes of other meningo-encephalitis causes identified a paucity of studies and very few data on morbidity and impairment[38,48]. This is indicative of the low value placed on the collection of long-term meningo-encephalitis outcomes, especially impairment/disability data. Very few studies conducted systematic screening for impairments with validated tools, which may lead to misclassifications and underreporting. Indeed, the two studies that systematically assessed the patients with validated tools found much higher proportions of impairments compared to the others (impaired cognition in 41% of HIV-CM survivors[33] and hearing impairment in 31% of non-HIV-CM survivors[35]). Therefore, future CM research should not only focus on short-term medical outcomes, but also on longer-term impairments,

disability and quality of life. WHO definitions of disability[9] as well as validated neurocognitive, sensorial and quality of life assessment tools should be used as in studies of other forms of encephalitis[38,48].

Implication for rehabilitation

Despite the limited amount of evidence, it is alarming to see that at one year, 20% to 70% of survivors have long-term impairments limiting cognitive, motor, visual and/or hearing function in both HIV and non-HIV-CM patients in both LMICs and HICs. And, while research and guidelines focus mainly on the management of the acute phase of CM to prevent acute mortality[49], little attention is given to long-term follow-up and the identification of, and support for, impairments and disability. Therefore, ensuring access to physical, occupational, sensorial and cognitive rehabilitation services is as important as enhancing prevention by fluconazole pre-emptive treatment and access to ART and AmB-based therapy[9]. While these services are routinely accessible in developed countries, they are limited in LMICs due to a lack of rehabilitation personnel and high costs[50]. For this reason, the development and adaptation of context specific rehabilitation models should be one of the global research priorities for CM and CNS infections, as already advocated by John et al.[50].

Limitations

In addition to the limitations related to the quality and heterogeneity of included studies, our review has some external limitations. Our inclusion and exclusion criteria might have restricted the panel of literature found. Eleven studies have been excluded because they included patients aged less than eighteen

(Supplementary Table 3). Moreover, the chosen threshold of quality is arbitrary as the CASP group has yet to propose a validated scoring system. This has led to exclusion of an additional eleven articles in order to decrease the risk of inaccurate conclusions (Supplementary Table 3). Nevertheless, each article excluded due to age or quality was carefully examined and its exclusion impact assessed. Subsequently, one article was then reintroduced in our review based on this assessment[16].

CONCLUSION

CM has an important long-term impact on mortality and disability. Nevertheless, the quality of evidence is limited, and future CM research should not only focus on short-term medical outcomes, but also on the longer-term mortality, impairments, disability and quality of life. In the meantime, fluconazole pre-emptive treatment, early diagnosis, as well as improved access to timely effective combined antifungal therapy should be implemented to prevent CM mortality and its long-term consequences. Additionally, early detection of impairments and access to rehabilitation services will improve CM-infected survivors' quality of life.

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TABLES:

Table 1: Overview of the 25 articles included in this review

	Article	Setting	Design	Sample size - number	Mean Age (except when mentioned) – years (IQR)	Information on		
						Mortality	Sequelae/Impairments	Disability/quality of life
HIV-CM								
1	Bicanic (2008)[19]	LMIC (South Africa)	RCT	64 (30 in AmB 0.7mg arm, 34 in AmB 1 mg arm)	33 (28-38)			
2	Day (2013)[16]	LMIC (Vietnam)	RCT	298 (99 in AmB arm, 100 in AmB+5-FC arm, 99 in AmB+Fluconazole arm)	Median: 28 (24-31) (Children 14-18 years old included)			
3	Makadzange (2010)[28]	LMIC (Zimbabwe)	RCT	54 (28 in early ART arm and 26 in late ART arm)	37 +/-7.7			
4	Boulware (2014)[20]	LMIC (South Africa)	RCT	177 (88 in early ART arm and	Median: Early ART: 35 (28-			

		LMIC (Uganda)		89 in late ART arm)	40) Late ART: 36 (30-40)			
5	Kambugu (2008)[18]	LMIC (Uganda)	PC	44	36 (31-42)			
6	Bicanic (2007)[26]	LMIC (South Africa)	PC	54 (36 ART Naïve, 18 ART experienced)	34 (29-39)			
7	Bicanic (2009)[29]	LMIC (South Africa)	PC	100 (65 survived for analysis on IRIS : 11 in IRIS arm and 54 in non-IRIS arm))	>18 years			
8	Boulware (2010)[30]	LMIC (Uganda)	PC	101 (56 in IRIS arm, 45 in non-IRIS arm)	?			
9	Butler (2012)[17]	LMIC (Uganda)	PC	189	36.2 (SD +/-8.8)			
10	Rothe (2013)[27]	LMIC (Malawi)	PC	60	Median: 32 (29-39)			
11	Chaiwarith (2014)[25]	LMIC (Thailand)	RC	79	35.1 +/-7.2			

1 2	Carlson (2014)[33]	LMIC (Uganda)	PC	78	35 +/-8			
1 3	Lizarazo (2012)[36]	LMIC (Columbia)	RC	63	34 +/-9.2			
1 4	Jarvis (2014)[21]	LMIC (South Africa)	PC	263	Median: 34 (29-39)			
1 5	Lanoy (2011)[22]	HIC (France)	PC	1020	<30y: 16% 30-40: 50% 40-50: 24% >50: 11%			
1 6	Mathiesen (2012)[24]	HIC (Denmark)	RC	45	40 (33-46)			
1 7	Cachay (2010)[42]	HIC (USA)	RC	82	38 (19-57)			

	Article	Setting	Design	Sample size (number)	Age (years)	Information on		
						Mortality	Sequelae/Impairments	Disability/quality of life
HIV and non-HIV-CM								
18	Liao (2012)[23]	HIC (Taiwan)	RC	72	<u>HIV:</u> 19 <u>non-HIV:</u> 53	<u>HIV:</u> 33.3 +/-7.4 <u>non-HIV:</u> 55.3 +/-15.7		
19	Lee (2011)[37]	HIC (Taiwan)	RC	88	<u>HIV:</u> 37 <u>non-HIV:</u> 51	<u>HIV:</u> 38.19 +/-12.12 <u>non-HIV:</u> 59.57 +/-14.17		
Non-HIV-CM								
20	Zhu (2010)[32]	LMIC (China)	RC	154	Median: 38.5			

2 1	Wang (2005)[35]	HIC (Taiwan)	PC	26	No Hearing loss group: 47 +/-21 Hearing loss group: 57 +/-17			
2 2	Phillips (2015)[34] (<i>C. gattii</i>)	HIC (British Columbia, Canada)	RC	47	Median: 50 (range 21-89)			

	Article	Setting	Design	Sample size (number)	Age (years)	Information on		
						Mortality	Sequelae/Impairments	Disability/quality of life
234	Chen (2012)[11] and (2013)[31] (<i>C. gattii</i>)	HIC (Australia)	RC	73	?			
25	Sun (2009)[51]	HIC/MIC (USA, France, Spain, Canada, India)	PC	75	51.5 (43-60)			

RCT: Randomized Control Trial, PC: Prospective Cohort, RC: Retrospective Cohort.

HIC: High Income Countries. LMIC: Low and Middle Income Countries

Table 2: Long-term mortality:

Study	Setting	Design	Sample size	<i>C. gattii</i> (CG) <i>C. neoformans</i> (CN)	Mortality % –[95% CI] ^a (Numbers) (PD or SA) ^b	Quality's score
HIV-CM						
>3months-4months after diagnosis or induction treatment						
Liao (2012)[23]	HIC (Taiwan)	RC	19	No data	5.3% (1/19) (PD) d90	54%
Lee (2011)[37]	HIC (Taiwan)	RC	37	No data	29.7% (11/37) (PD) d90	58%
Chaiwarith (2014)[25]	LMIC (Thailand)	RC	79	CN (92%)	32.4% (24/74) (PD) d90	65%
Lizarazo (2012)[36]	LMIC (Columbia)	RC	63	CN	54% (SA) d120	50%
6 months after diagnosis or induction treatment						
Cachay (2010)[42]	HIC (USA)	RC	82	No data	6.1% (5/82) (PD)	64%

^a No 95% CI mentioned if not available

^b PD: Mortality expressed as a Percentage of Death at the time point (lost to follow-up excluded when known), or SA: Survival Analysis (probability of mortality at a time point, lost to follow-up censored)

Bicanic (2008)[19]	LMIC (South Africa)	RCT	64	No data	32% (PD)	55%
Boulware (2014)[20]	LMIC (South Africa – Uganda)	RCT	177	No data	37.9% (67/177) (PD)	82%
Day (2013)[16]	LMIC (Vietnam)	RCT	298	CN (VNI)	45% (132/291) (PD)	82%
Butler (2012)[17]	LMIC (Uganda)	PC	189	No data	52% [45-59] (SA)	58%
Bicanic (2009)[29]	LMIC (South Africa)	PC	100	No data	53% (53/100) (PD)	54%
Kambugu (2008)[18]	LMIC (Uganda)	PC	44	No data	59.1% (26/44) (PD)	73%
1 year after diagnosis or induction treatment						
Lanoy (2011)[22]	HIC (France)	PC	1020	No data	50% [45-54] (SA) during the Pre-cART period: 92-95 24% [18-29] (SA) during the Early cART period: 96-98 17% [12-22] (SA) during the Late cART period: 99-04	64%
Liao (2012)[23]	HIC (Taiwan)	RC	19	No data	22.2% (4/18) (PD)	54%
Mathiesen (2012)[24]	HIC (Denmark)	RC	45	No data	44.2% (SA) during the full period 88-08	81%

					23% (SA) during the cART period 97-08	
Boulware (2010)[30]	LMIC (Uganda)	PC	101	No data	27.7% (28/101) but first weeks deaths excluded	77%
Boulware (2014)[20]	LMIC (South Africa – Uganda)	RCT	177	No data	39.5% (70/177) (PD)	82%
Bicanic (2008)[19]	LMIC (South Africa)	RCT	64	No data	40% (PD)	55%
Jarvis (2014)[21]	LMIC (South Africa)	PC	263	No data	41% (PD)	57%
Chaiwarith (2014)[25]	LMIC (Thailand)	RC	79	No data	52.2% (36/69) (PD)	65%
Butler (2012)[17]	LMIC (Uganda)	PC	189	No data	55% [38-52] (SA)	58%
Kambugu (2008)[18]	LMIC (Uganda)	PC	44	No data	59.1% (26/44) (PD)	73%
Bicanic (2007)[26]	LMIC (South Africa)	PC	54	No data	64.7% (33/51) (PD)	69%
Rothe (2013)[27]	LMIC (Malawi)	PC	60	No data	78% [64-86] (SA)	69%

Study	Setting	Design	Sample size	<i>C. gattii</i> <i>C. neoform</i>	Immune Status	Mortality %age –[95% CI] ^c (Numbers) (PD or SA) ^d	Quality ^e score
Non- HIV-CM							
>3months-4months after diagnosis or induction treatment							
Sun (2009)[51]	HIC (USA, Canada, France, Spain) + India	PC	75	No data	100% Solid Organ Transplant	18.7% (14/75) (PD) d90	69%
Liao (2012)[23]	HIC (Taiwan)	RC	53	No data	100% with underlying conditions	30.8% (16/52) (PD) d90	54%
Lee (2011)[37]	HIC (Taiwan)	RC	51	No data	92% with underlying conditions	33.3% (17/51) (PD) d90	58%
1 year after diagnosis or induction treatment							
Chen (2012)[11]	HIC (Australia)	RC	73	CG	28% with underlying conditions	13% (10/73) (PD)	68%

^c No 95% CI presented if not available

^d PD: Mortality expressed as a Percentage of Death at the time point (lost to follow-up excluded when known), or SA: Survival Analysis (probability of mortality at a time point, lost to follow-up censored)

Phillips (2015)[34]	HIC (Canada)	RC	47	CG	45% with underlying conditions	27.7% (13/47) (PD)	64%
Zhu (2010)[32]	LMIC (China)	RC	154	No data	33% with underlying conditions	28.7% (41/143) (PD)	62%
Liao (2012)[23]	HIC (Taiwan)	RC	53	No data	100% with underlying conditions	42.3% (22/52) (PD)	54%

RCT: Randomized Control Trial, PC: Prospective Cohort, RC: Retrospective Cohort.

HIC: High Income Countries LMIC: Low and Middle Income Countries

D90: at day 90 after diagnosis or induction treatment; D120: at day 120 after diagnosis or induction treatment

Table 3: HIV-CM predictive factors of long-term mortality identified by the review

Predictive factors	Number of studies	Studies	Design Quality score	Outcome measure of association (95%CI) and/or p-value if informed by the authors
Protective factors				
ART vs no ART at induction	2	Mathiesen (2012)[24] <i>Lanoy (2011)[22]</i>	RC 81% PC 69%	Rate Ratio ^a (Cox) for 1 year mortality=0.22 (0.06-0.77) p=0.018 <i>1-year mortality (Kaplan-Meier) in pre-ART = 50% (45-54) vs 17% (12-22) in late-ART</i>
Early ART initiation vs late ART initiation after induction (for ART naïve patients)	2	Boulware (2014)[20] Makadzange (2010)[28]	RCT 82% RCT 50%	ART initiation at 2 wks vs 5 wks: HR ^e (Cox) for 11 months mortality = 1.66 (1.03-2.68) p=0.04 ART initiation at <72h vs 10 wks: HR ^f (Cox) for 3 years mortality = 2.85 (1.1-7.23) p=0.031

^e Not adjusted because RCT with similar characteristic in both arms (p>0.1 for all characteristics)

^f Adjusted on age, sex, CSF CrAG titer, CD4 level

AmB based bitheraPy vs monotherapy	1	Day (2013)[16] <i>Bicanic (2008)[19]</i>	RCT 82% RCT 55%	AmB+5FC vs AmB: HRa ^l (Cox) for 6 months mortality = 0.56 (0.36-0.87) p=0.01 AmB+5FC vs AmB+Fluconazole: HRa ^l (Cox) for 6 months mortality = 0.55(0.35-0.88) p=0.01 <i>AmB 0.7mg/kg vs AmB 1 mg/Kg (+ 5FC) for 6 months and 12 months mortality: no difference (Fisher exact test – no data neither p-value given)</i>
Risk factors				
Older Age	1	Mathiesen (2012)[24]	RC 81%	Rate Ratio a ^g (Cox) for 1 year mortality for each year increase (in age) = 1.05 (0.99-1.11) p=0.054
Altered neurology at induction vs no altered	2	Chaiwarith (2014)[25]	RC 65%	Ora ^h (Logistic) for 1 year mortality = 5.27 (1.26-24.05)

^g Variables included in the model: age, sex, mental status, CD4, time-updated initiation of ART, antifungal regimen, flucytosine induction, nationality, time-period (88-96/97-08), hospital centre (variables with p<0.1 were included in the model)

^h Variables included in the model (with p<0.10 in univariate) are altered mental status, seizures, CSF CrAg titer, year of diagnosis (2005-2010)

neurology (with different items according to studies)		Day (2013)[16]	RCT 82%	HRa ¹ (Cox) for 6 months mortality =2.30(1.57-3.36) p<0.001
Baseline elevated fungal burden (CSF CrAg titres or fungal load)	2	Chaiwarith (2014)[25] Day (2013)[16]	RC 65% RCT 82%	ORa ^{1b} (Logistic) elevated CrAg for 1 year mortality = 7.08 (1.62-31) HRa ¹ (Cox) elevated fungal load for 6 months mortality=1.33 (1.08-1.65) p=0.01 for each increase of 1 log ₁₀ CFU/mm ³

¹ Adjusted on Age, Sex, Headache, Fever, Neck stiffness, Seizure, Glasgow coma score, Cranial-nerve palsy, papilledema, CSF opening pressure>18 cm H₂O, CSF WCC, CSF glucose, plasma glucose

Low CD4 at induction (<20 vs >20)	1	Liao (2012)[23]	RC 54%	ORa ^l (Logistic) for > 1 year death or relapse=18(1.19-71.46) p=0.037
<i>Contradictory results</i>				
IRIS vs no IRIS during the CM episode	3	Boulware (2010)[30] <i>Bicanic (2009)[29]</i> <i>Boulware (2014)[20]</i>	PC 77% <i>PC 54%</i> <i>RCT 82%</i>	HRA ^k (Cox) for 1 year mortality = 2.3(1.1-1.51) p=0.04 <i>No association (Fisher exact test) as 36% of 6-months mortality in IRIS vs 26% in non-IRIS, p=0.49</i> <i>IRIS in early ART vs late ART: 20% [17/87] and 13% [9/69], respectively, p=0.32 (while mortality significantly higher in early ART)</i>

Evidence based on multivariate analysis

Evidence based on univariate analysis

^l Variables included in the model: clinical, laboratory variables at presentation + antifungal treatment given.

^k Adjusted on baseline CD4, all other characteristic similar in both groups (BMI, time from CM to ART, Opportunistic infection, Baseline CD8, Baseline and subsequent VL, subsequent CD4, Eosinophils, initial serum CrAG titer median)

PC: Prospective Cohort, RC: Retrospective Cohort, RCT: Randomized Control Trial

Table 4: Non-HIV-CM predictive factors of long-term mortality identified by the review

Predictive factors	Number of studies	Studies	Design Quality score	Outcome measure of association (95%CI) and/or p-value if informed by the authors
Protective factors				
AmB based therapy	2	Zhu (2010)[32] Sun (2009)[51]	RC 62% PC 69%	Non AmB-based vs AmB based: HR ^a (Cox) for 1-year mortality = 8.87(3.53-22.25) p<0.001 AmB lipid vs AmB deoxylate: OR ^a (Logistic) for 3 months mortality=0.11(0.02-0.57) p=0.008
Risk factors				
Delayed diagnosis vs no delayed	1	Zhu (2010)[32]	RC 62%	HR ^a (Cox) = 6.3(2.41-16.53) p<0.001
Older Age	1	Zhu (2010)[32]	RC 62%	No data shown

^a Variables included in the model: age, sex, time to diagnosis>4months, pulmonary crypto, AID, hematological malignancy, solid malignancy, corticotherapy, transplantation, healthy, altered mental status, coma, seizure, cerebral herniation, non AmB based initial therapy, inclusion of 5FC, intrathecal AmB treatment, ommaya implantation

Altered neurology at induction vs no altered neurology (with different definitions according to studies: focal neurologic signs, hydrocephalus, Low Glasgow score, herniation)	3	Zhu (2010)[32] Liao (2012)[23] Chen (2012) C. Gattii[11]	RC 62% RC 54% RC 68%	HRA ^l (Cox) = 8.08(2.96-16.95) p<0.001 ORa ^m (Logistic) for > 1 year death or relapse=8.7(2.23-28.98) p=0.003 1 year mortality 19% in abnormal neuro vs 3% in normal neuro, (Fisher exact test) p=0.05
Baseline elevated fungal burden (CSF CrAg titres)	1	Liao (2012)[23] Chen (2012) C.Gattii	RC 54% RC 68%	>1/512 vs <1/512: ORa ^m (Logistic) for > 1 year death or relapse=16.2(1.37-192.02) p=0.027 >1/256 vs <1/256: Ora for 1 year mortality=1.8(1-26) p=0,05
Fungemia vs no fungemia in SOT	1	Sun (2009)[51]	PC 69%	ORa ⁿ (Logistic) for 3 months mortality= 10.6 (2.08-54.55) p=0.004
Renal failure vs no renal failure in SOT	1	Sun (2009) [51]	PC 69%	ORa ⁿ (Logistic) for 3 months mortality= 4.61(1.02-20.8) p=0.047
<i>Contradictory results</i>				

^m Variables included in the model: clinical, laboratory variables at presentation + antifungal treatment given.

ⁿ Variables included in the model (with p<0.20 in univariate) are CNIs, renal failure at baseline, abnormal mental status, fungemia, receipt of lipid AmB (backward strategy)

Underlying conditions vs healthy	2	Chen (2012) C Gattii [11] Zhu (2010)[32]	RC 68% RC 62%	1 year mortality 26% in predisposed vs 7.4% in healthy (Log-rank test) <i>p</i> =0.03 1-year mortality 26.5% in predisposed vs 29.8% in healthy (Fisher exact test) <i>p</i> =0.69
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Evidence based on multivariate analysis

Evidence based on univariate analysis

PC: Prospective Cohort, RC: Retrospective Cohort, RCT: Randomized Control Trial, SOT: Solid Organ Transplant

FIGURES:

Figure 1: Flow diagram of the article inclusion/exclusion process (inspired from PRISMA 2009 Flow Diagram[14]).

Figure 2: Forest plot of proportion of one-year mortality in HIV-CM according to induction therapy received.

Legends of figure 2:

4 studies are excluded from this forest plot:

- Boulware et al (2010) study because the mortality is probably under-estimated[30]. The patients were included after ART initiation at a median time from CM diagnosis of 34 days (IQR: 24-41), missing all death happening during the first weeks.

- Lanoy et al (2011)[22], Liao et al (2012)[23] and Mathiesen et al (2012)[24] because no information was available or extractable for the treatment received by the patients infected by HIV or for the dichotomised periods.

In the Bicanic 2007 study[26], 92% of patients were treated by AmB monotherapy and 8% of patients were treated by fluconazole monotherapy.

Figure 1

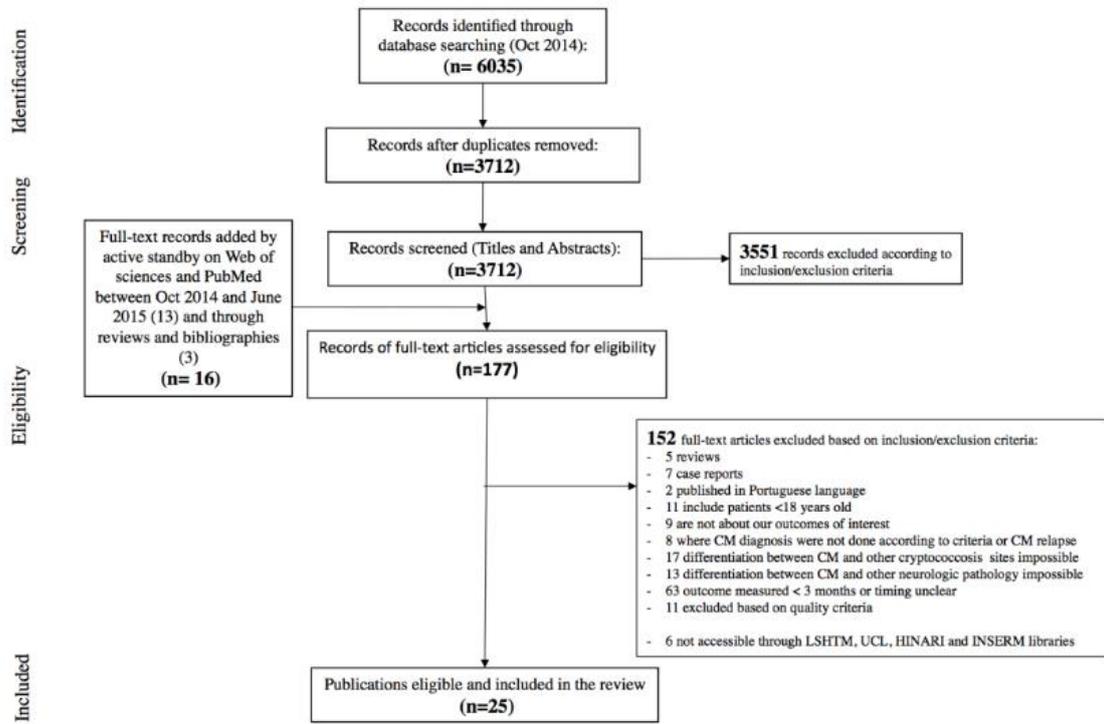
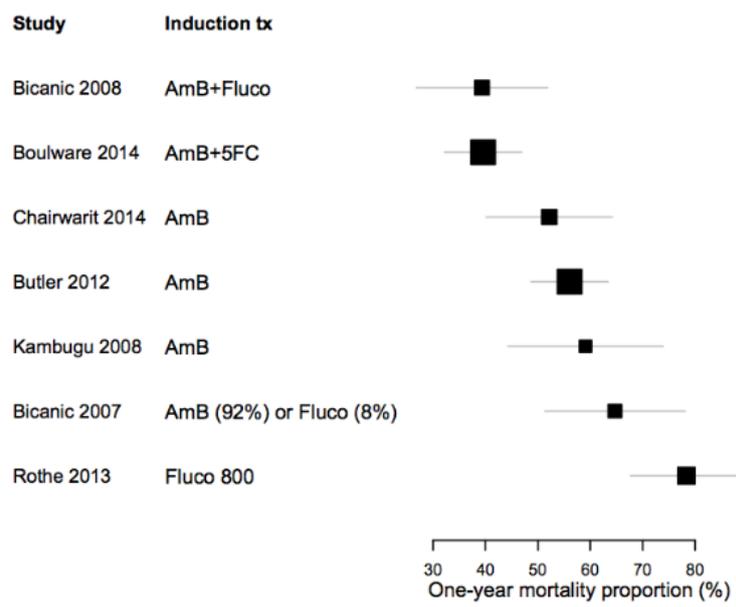


Figure 2



2.7. Cryptococcal meningitis as a policy priority: advocacy

2.7.1. Draft article 7: Published in PLoS Neglected Tropical Diseases

VIEWPOINTS

Cryptococcal meningitis: A neglected NTD?

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Although HIV/AIDS has been anything but neglected over the last decade, opportunistic infections (OIs) are increasingly overlooked as large-scale donors shift their focus from acute care to prevention and earlier antiretroviral treatment (ART) initiation. Of these OIs, cryptococcal meningitis, a deadly invasive fungal infection, continues to affect hundreds of thousands of HIV patients with advanced disease each year and is responsible for an estimated 15%–20% of all AIDS-related deaths [1, 2]. Yet cryptococcal meningitis ranks amongst the most poorly funded “neglected” diseases in the world, receiving 0.2% of available relevant research and development (R&D) funding, according to Policy Cures’ 2016 Global Funding of Innovation for Neglected Diseases (G-Finder) Report [3, 4].

Although cryptococcal meningitis is not formally recognised by the World Health Organisation (WHO) or *PLOS Neglected Tropical Diseases* (*PLOS NTDs*) as a neglected tropical disease (NTD), it is listed in the G-Finder report, as it disproportionately affects people in low- and middle-income countries (LMICs), with market failure evident for existing essential antifungal medicines and an urgent need for new, effective, and less toxic medicines. *PLOS NTDs* defines NTDs as a “group of poverty-promoting chronic infectious diseases, which primarily occur in rural areas and poor urban areas of LMICs” [5] and according to the WHO, NTDs are “a proxy for poverty and disadvantage”, have “an important impact on morbidity and mortality”, and are relatively “neglected by research” [6]. Although the greatest burden of cryptococcal disease is undoubtedly related to HIV, we demonstrate herein that cryptococcal meningitis meets both the WHO and *PLOS NTDs* definitions of an NTD,

as the disease (1) disproportionately affects populations in poverty and causes substantial morbidity and mortality, (2) primarily affects populations living in tropical and subtropical areas, (3) is immediately amenable to broad control, elimination, or eradication, and (4) is neglected by research [7].

Morbidity, mortality, and poverty associated with cryptococcal meningitis

As the most common cause of meningoencephalitis in sub-Saharan Africa and Southeast Asia, cryptococcal meningitis disproportionately affects populations in some of the most economically disadvantaged regions of the world [1–3]. Mortality rates remain high, ranging between 20% and 60% with treatment and up to 100% without, affecting the most economically productive age groups [1, 8–11]. Significant morbidity is often encountered, with severe headaches occurring weeks to months before presentation and potential long-term sequelae including blindness and deafness [12–14]. Such morbidity and mortality places a substantial economic burden on patients and their families, resulting in lost productivity and wages as well as patient-transportation and medical costs. Effective treatments are intensive, expensive (for example, where available, first-line induction therapy with amphotericin B therapy costing over US\$200 in Uganda), and available only in hospitals (due to the need for intravenous [IV] administration of amphotericin B and need for close laboratory monitoring for toxicities) [15, 16].

A disease of the tropics and subtropics

ART scale-up is ultimately the key to reducing cryptococcal meningitis mortality, as has been demonstrated in Europe and North America [17, 18]. However, the burden of morbidity and mortality in LMICs has remained largely unchanged and shows no sign of abating [2, 19–22]. The number of severely immunosuppressed patients not yet accessing ART has remained relatively constant and, in some instances, even increased [23–28]. Data from a recent meta-analysis estimated that over half of patients in Africa had progressed to AIDS with CD4 counts <200 cells/mm³ by the time they initiated ART [29], despite over a decade of ART program implementation and scale-up.

While new WHO guidance on a strategy of universal HIV testing and treatment has the potential to prevent cryptococcal disease, poor retention in care and ART failure currently have a significant impact on the incidence of cryptococcal meningitis in Africa. In many hospitals, over half of cryptococcal meningitis cases now occur in patients previously prescribed ART, either in the early months following initiation of ART as unmasking of subclinical infection or, more commonly, later, as a consequence of nonadherence and/or ART failure [28, 30]. There is no sign of a reduction in the high mortality rates for HIV-associated cryptococcal meningitis, driven by late presentation, delayed diagnosis, and inadequate access to and efficacy of current antifungal medicines [31]. Though a number of HIV programs in South and Southeast Asia have achieved some success in terms of ART retention and early detection, the proportion of patients presenting to care with blood samples testing positive for cryptococcal antigen (CrAg) rival those seen in many settings in Africa, demonstrating that cryptococcal meningitis still presents a significant problem, even if absolute numbers of cases are lower [32–35].

Lack of R&D

A common misconception is that funding for cryptococcal meningitis comes under the umbrella of HIV. In reality, cryptococcal meningitis has fallen into a research and policy gap [4]. The research gap is most evident in drug development, as only 3 antifungal medicines—

amphotericin B, flucytosine (5-FC), and fluconazole—are currently used for the treatment of cryptococcal meningitis, all of which are decades old [21, 31]. Current WHO guidelines recommend 2 weeks of amphotericin B and 5-FC as the initial intensive induction phase, followed by a step-down to fluconazole for the consolidation and maintenance phases of treatment [36, 37]. However, this gold standard induction phase remains aspirational for most LMICs. Both amphotericin B and 5-FC are unavailable in the majority of LMICs, and the cheaper, more widely available fluconazole is an inadequate treatment for induction therapy [10]. In addition to patchy drug registration, amphotericin B is often not administered due to issues with toxicity (i.e., anaemia and renal impairment), poor laboratory monitoring facilities, cost barriers, and requirements for cold-chain shipment/storage at 4°C [31, 38]. Although there are 3 stringent regulatory authority (SRA)-approved generic manufacturers of 5-FC (Meda Pharm [recently acquired by Mylan], Sigmapharm, Valeant), none are currently registered in Africa [31, 38, 39, 40] despite compelling data for 5-FC's use in combination with either amphotericin B or fluconazole during the induction phase of treatment (regimens both recommended in the latest WHO guidelines [8, 14, 36, 37, 40]). Instead, fluconazole is widely available, well tolerated, and used for all phases of cryptococcal treatment despite clear evidence that fluconazole alone is a poorly effective medicine when used during the intensive induction phase, with 10-week mortality consistently >50% [8, 9, 10, 41].

R&D into the manufacture of new or improved therapies is currently inadequate. In 2015, R&D for cryptococcal meningitis totalled US\$5.8 million (just over 1% of the R&D budget afforded to tuberculosis [US\$567 million]), placing it in the bottom tier of neglected diseases, receiving support essentially from 3 public health funders: United States National Institutes of Health (NIH), United Kingdom Medical Research Council (MRC), and Australian National Health and Medical Research Council [3]. Funding for HIV/AIDS does not currently encompass OIs such as cryptococcal meningitis and, as yet, neither has it fallen under the umbrella of NTD funding. Very few new drugs for cryptococcal meningitis are in the human safety and dose-finding trial stage of development. VT-1129 and VT-1598 (Viamet Pharmaceuticals) are long-acting, azole-like compounds developed with assistance from the NIH's Therapeutics for Rare and Neglected Diseases programme. In vitro and animal models have demonstrated excellent activity and good oral pharmacokinetics, safety profile, and central nervous system penetration (www.viamet.com/pipeline.asp [42]). In addition, T-2307, which targets the fungal mitochondrial membrane, has completed initial human safety and dose-finding trials in the US (www.toyama-chemical.co.jp/en/rd/pipeline/index.html [43]). Oral formulations of amphotericin B (Matinas Biopharma) and compounds with activity against *Cryptococcus neoformans* (including AN 2690 [Anacor Pharmaceuticals], ASP 2397 [VICAL], and Ar 12 [Arno Therapeutics]) are currently only in preclinical stages of development. No additional new anti-fungal medicines have been developed for the treatment of cryptococcal meningitis in over 2 decades.

Strategies for control of cryptococcal meningitis

Despite the challenges, 2 strategies for the control and treatment of cryptococcal meningitis exist that together could make a significant impact: screen-and-treat programs for CrAg and delivery of short-course improved therapies [6]. CrAg screening of severely immunosuppressed patients (typically with CD4 counts ≤ 100 cells/mm³), which is recommended by the WHO and the President's Emergency Plan for AIDS Relief (PEPFAR), combined with pre-emptive antifungal treatment can prevent or detect at an early stage a significant proportion of cryptococcal meningitis cases, as the antigen can be detected in blood for weeks to months prior to the development of clinical disease. There are sensitive and specific CrAg lateral flow

assays (CrAg LFAs) that can be used for diagnosis either in the laboratory or at the point of care. The strategy is highly cost-effective and has recently shown a mortality benefit when combined with increased adherence support and early detection of tuberculosis (TB) [16, 44]. Although CrAg screening has been adopted into policy by over 20 countries, few have committed resources to implementing such programs; attention brought to cryptococcal meningitis as an NTD could help move such policy to action and begin saving lives.

With regard to treatment, optimized, sustainable regimens using existing antifungal medicines, drug development, and testing of repurposed drugs could significantly reduce the case fatality rate. Simplified treatment regimens consisting of short-course combination amphotericin B therapy and a purely oral combination of fluconazole and 5-FC are currently being tested in African LMICs with results expected in 2017 (ACTA trial, ISRCTN45035509). Liposomal formulations of amphotericin B offer a far better-tolerated alternative to traditional formulations. The AMBITION-cm trial is comparing the efficacy of 1 high dose of liposomal amphotericin in combination with fluconazole for HIV-associated cryptococcal meningitis (AMBITION-cm, ISRCTN10248064). A few intermittent doses could prove cost-effective if hospital admission duration was reduced, and there is precedent for efforts to increase access. In September 2016, Gilead extended a donation program to the WHO to make an additional 380,000 vials of liposomal amphotericin available at cost to continue treating patients with visceral leishmaniasis over 5 years [45]. If Gilead's program were expanded to cover cryptococcal meningitis, this could make improved, simplified treatment of meningitis far more accessible to resource-limited populations where disease burden is highest.

Repurposing of established drugs has also been applied to the search for new medicines [46]. This approach may expedite translation into clinical practice and reduce drug-development costs. Sertraline and tamoxifen are 2 such drugs that are fungicidal against *C. neoformans*, with good central nervous system penetration and synergy with fluconazole [30]. Both drugs are being studied separately as new adjunctive therapies for HIV-associated meningitis.

Lastly, raised intracranial pressure affects approximately one third of patients, and managing this common, debilitating, life-threatening complication improves patient outcomes [47, 48]. However, access to manometers, used to measure intracranial pressure, remains inadequate in LMICs and needs to be addressed alongside validation of simple measures to determine flow rates of cerebrospinal fluid (CSF) through spinal needles [49].

Consideration for inclusion in NTD list

Whilst debate continues as to whether cryptococcal meningitis should be listed as an NTD, affected persons in LMICs remain neglected, with opportunities missed to both prevent disease and provide effective treatment to reduce mortality. Stating that increased ART coverage alone will obviate the problem of HIV-associated cryptococcal meningitis in LMICs is not tenable; the evidence, with striking data from Botswana of ongoing cryptococcal meningitis cases despite excellent ART coverage, shows that this has not been the case to date [22]. Patients with advanced HIV-related cryptococcal meningitis will continue to present to hospitals in LMICs for the foreseeable future.

In 2013, the Cryptococcal Meningitis Action Group (CryptoMAG) was formed to improve access to diagnostic tests and essential antifungal medicines and to disseminate clinical best practices for treatment and prevention in LMICs. The debate over whether or not cryptococcal disease is an NTD detracts from cryptococcal meningitis being both HIV related and also urgently needing the interventions (funding, policy drives, and drug pipelines) from which NTDs benefit. We therefore call on the global health community, *PLOS NTDs*, UNITAID, The Global Fund, and WHO to declare cryptococcal meningitis an NTD and press for urgent

funding and policy drives to target optimisation and rollout of CrAg-screening programs, access to 5-FC and liposomal amphotericin B, and new drug development.

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3. General Discussion

During the period spanning April 2014 and October 2017, we carried out as part of a post-graduate PhD project, a piece of work entitled "*Epidemiology and optimal management of cryptococcal meningo-encephalitis associated with AIDS in Cameroon*". This project was aimed at contributing in the generation primary and secondary data which could serve as template for guiding local policy on the optimal management of HIV-associated cryptococcal meningitis in Cameroon. The present PhD project was supported financially by the ANRS, was enrolled at the Paris Descartes University in France, supervised by Pr. Olivier Lortholary in the Molecular Mycology Unit of Institut Pasteur of Paris headed by Pr. Françoise Dromer.

This piece of work for which there was a diverse network of fruitful international collaborations, was designed on a model which evaluated the effects on patient outcome, of optimal interventions tailored at different evolutive stages of the spectrum of HIV-associated cryptococcosis. This spectrum was defined from onset of severe immune depression (predisposition to cryptococcosis) through asymptomatic and symptomatic cryptococcosis (in this case CM) to the long-term period following the treatment of a first episode of confirmed CM. As such, the milestones of this continuum of interventions were

- Prevention (targeted pre-emptive oral fluconazole after CrAg screening)
- Diagnosis (asymptomatic and symptomatic disease)
- Treatment (most effective and tolerated inductive treatment for the African context)
- Prognosis (long term disability and mortality beyond three months of CM treatment)
- Policy (availability of interventions for optimal management).

The findings of this work will be discussed based on the chronology of these milestones of CM spectrum without necessarily considering the timelines of the different studies in which the interventions were evaluated.

The objectives of this project were based on operational studies requiring international collaboration and individual contributions. For these, we played the following roles

1. As principal investigator “South” of the ANRS 12 312 PreCASA study, we were responsible for
 - Designing the study, applying and obtaining funding
 - Communicating with hospital administration, ministry of health and ethics committee for required administrative and ethical clearance.

- Recruiting, training, coordinating and managing the study team with whom we did patient recruitment, follow up and data collection
 - Monitoring, management and analysis of study data
 - Monitoring and coordination of reporting of adverse events
 - Communicating with the study sponsor.
2. As co-investigator in the ANRS 12275 ACTA trial we were responsible for
 - Setting-up a second study site at the Douala General Hospital
 - Communicating with hospital administration for clearance
 - Recruiting, training, coordinating and managing the study team with whom we did patient recruitment, follow up and data collection
 - Assuring data quality control and report of adverse events.
 - Communicating study site updates to international teams during yearly report meetings.
 - Designing and carrying out secondary analysis of trial data on the safety and tolerability of AmB used during the trial.
 3. As main review author of the systematic reviews and meta-analyses of CrAg detection, we were responsible for
 - Designing the study, writing the protocol
 - Communicating and coordinating the activities of the review team
 - Screening citations obtained following electronic database searches,
 - Extracting and analysing data.
 4. As co-author in the systematic review on long term outcome of CM, we participated in
 - Proofreading the review protocol
 - Data extraction and proofreading of the final manuscript
 5. As co-author on the advocacy study we contributed in drafting and proofreading the write-up

Overall, I strongly hope that the data obtained during this PhD thesis will be helpful in optimising the management of HIV related CM in Cameroon. Indeed, till date, the burden of CM as a major cause of adult meningitis has been sparingly described there [64]. Though the second major cause of CNS associated diseases of which at least one of two affected patients dies [91], there are no local policies for the prevention, diagnosis and treatment of HIV-related CM. Therefore, the first interventions on our model of optimal management to be evaluated was systematic pre-ART screening and pre-emptive fluconazole-based therapy as a strategy to

reduce the burden of HIV-associated CM as recommended by the WHO [72]. Prior to carrying out systematic CrAg screening in our milieu, we needed lessons from other settings with routine practice especially as this was first experience of in Cameroon.

In collaboration with the Cochrane team of Amsterdam and Necker Hospital Paris, we did a systematic review and meta-analysis on the effect of systematic CrAg screening and pre-emptive fluconazole on the incidence of CM and CM-related mortality within the first year of ART.

We found that a median of 6% of patients with <100 CD₄ cells/ml were CrAg positive and that with no fluconazole offered to CrAg positive patients following screening, the incidence of CM was more than 50 folds higher in CrAg positive patients compared to those who were CrAg negative. When CrAg positive patients were offered fluconazole at any dose (200 – 800 mg/day) even at physician's discretion, incident CM risk in CrAg positive though still 15 folds higher than in CrAg negative patients, had reduced more than 70% of what is observed when no fluconazole is offered. In studies which offered post screening LP to CrAg positive patients (to rule out asymptomatic CM) prior to starting fluconazole at 800 mg/day for a minimum of two weeks, CM risk dropped to a tenth of that of patients not receiving fluconazole but was still about 6-fold higher than in CrAg negative patients. However, looking at the confidence interval of the effect size estimate of post screening LP prior to high dose fluconazole, we found that in some CrAg positive patients, there was a 35% lower risk of CM than CrAg negative patients. Therefore, high dose fluconazole after post screening LP, compared to no fluconazole reduces CM risk by over 90% in CrAg positive patients. We postulate that this intervention could have reduced this risk even further if all CrAg positive patients accepted an LP because those who refused, might have had asymptomatic CM that contributed to the excess incidence of CM in CrAg positive patients. Therefore, our take home message from this SRMA was that pre-emptive therapy as presently recommended, offered to asymptomatic CrAg positive patients after post screening LP to rule out asymptomatic CM is effective in significantly decreasing the risk of incident CM in CrAg positive patients.

In a second approach and in collaboration with a team in St. Georges University of London (Thomas Harrison, Angela Loyse and Sile Molloy), a team in Yaounde Central Hospital (Charles Kouanfack, Leonella Mossiang), the ANRS site in Yaounde (Estelle Pasquier, Sylvie le Gac, Georges Mounpou, Eugenie Dipoue) and the Centre Pasteur of Cameroon (Marie Christine Fonkoua, Esther Sokeng), Olivier Lortholary and I designed a prospective study on systematic pre-ART CrAg screening patients presenting with less than 100 CD₄ cells/ml at the

Day Hospital of the Yaounde Central hospital. Initial results of this study were presented at the 10th International Conference on Cryptococcus and Cryptococcosis (ICCC) in Foz de Iguacu in Brazil from March 26th to 30th 2017 and was awarded a prize for oral presentation (ET).

CrAg screening was done with three tests of which two POC tests IMMY LFA (as recommended by WHO) and Biosynex CryptoPS (a new POC LFA-based with two test bands, a qualitative T1 and a semi-quantitative T2 band) and ELISA test (considered a reference test). Notwithstanding, only patients with serum positive to IMMY test were considered CrAg positive. Consequently, baseline serum CrAg was 7.5%, thus comparable to that in other settings. At point of screening, Biosynex CryptoPS was comparable to IMMY, both tests strongly agreed in serum in classifying patients as CrAg positive or negative but compared to ELISA, Biosynex CryptoPS was better than IMMY LFA in classifying false positives (Specificity: 98.3% vs. 96.6%).

In this cohort where CrAg screening was in both serum and urine, we decided to use a pragmatic and more pro-active approach whereby all patients with detectable CrAg (defined as positive IMMY LFA CrAg in serum and/or urine) were offered post screening LP and pre-emptive fluconazole especially as this was a first experience of CrAg screening in Cameroon. LP acceptance was moderate (62%) as seen in other settings. Baseline confirmed asymptomatic CM in serum CrAg positive patients was 45.5%, a similar burden to other settings suggesting that if we did not do post screening diagnostic LP, about 1 out of two of our patients would have been on sub optimal fluconazole treatment. At screening, all CM confirmed patients were T2 band positive with the Biosynex CryptoPS test and post screening antigen titres measured in all CrAg positive sera showed significantly higher titres in CM confirmed patients and a strong association at titre threshold >1:160. Biosynex CryptoPS was capable to identify patients with higher titres, thus predicted all cases of asymptomatic CM and so holds promise as an option for systematic CrAg screening and asymptomatic CM presumption in low LP uptake areas.

During the one year follow up period after ART initiation, there was no case of incident CM in our study population. This suggests that a comprehensive package comprising systematic CrAg screening, active post screening CM case finding, fluconazole pre-emptive therapy to asymptomatic CrAg positive patients and ART is effective in preventing incident CM during first year of ART.

Putting the findings of the SRMA on CrAg screening and our experience in Cameroon we conclude that: high prevalence of undiagnosed asymptomatic CM in CrAg positive patients is

a probable driver of the excess risk of CM in CrAg positive patients on high dose pre-emptive fluconazole giving a misleading impression that the strategy is not effective.

We therefore recommend systematic diagnostic LP to all patients screening CrAg positive prior to starting pre-emptive fluconazole.

Cryptococcal antigen titre at screening is a reliable surrogate for presumptive diagnosis of asymptomatic CM in patients screening CrAg positive. We recommend systematic CrAg screening with point of care test capable of identifying patients with higher titres and consideration of serum CrAg titres measurements in CrAg positive patients who refuse LP.

The take home messages from the previous two studies were that: asymptomatic CM is highly prevalent in serum CrAg positive patients; higher serum CrAg titre was strongly associated with prevalent asymptomatic CM at screening and incident symptomatic CM after ART initiation. Consequently, in the continuum of CM disease spectrum, for serum CrAg positive patients who missed the chance of asymptomatic CM diagnosis at screening, onset of symptomatic CM is only a matter of time, probably precipitated by IRIS following ART. In the Cameroon setting where pre-ART systematic CrAg screening is not routine practice, we hypothesised that within weeks following ART, patients may present with symptoms of CNS disease. We became interested in the role of serum CrAg screening in the diagnosis of CM in patients with CNS symptoms.

With our Amsterdam and Necker Hospital Cochrane team collaboration, we did a SRMA on the diagnostic accuracy of CrAg detection in serum and/or CSF as compared to Indian ink staining and/or culture of CSF of patients with clinical suspicion of CM. CrAg detection was considered done with any presently available commercial test kits irrespective of test principle (LA, LFA or ELISA). The studies included in the review showed that CM as diagnosed by CSF culture and Indian ink stain was prevalent in 46% of patients with CNS symptoms. Serum CrAg detection irrespective of the CrAg test principle perfectly agreed with CSF culture (100% sensitivity) in classifying patients with CM and misclassified only a few patients as false positive (96% specificity). This means that systematic serum CrAg screening in symptomatic patients will presumptively diagnose all CM cases and could identify patients for whom LP could be considered or for whom combination antifungal may be considered if LP not possible. Also, when we looked at CSF CrAg detection as a rapid diagnostic test compared to culture, the concordance was very high (sensitivity and specificity 99%) suggesting therefore that in

settings where LP can be done but Indian ink and culture cannot be delivered, CrAg screening in CSF is diagnostic of CM.

With these, we recommend that all patients presenting with CNS symptoms should be systematically screened for CrAg and a positive result considered presumptively diagnostic of CM, especially if previous screening was negative.

In parallel with ongoing former studies and as previously cited, I was strongly engaged in the “ACTA” trial in Douala. In Cameroon, like in many African settings, treatment of CM relies on high dose fluconazole due to lack of recommended treatment. More so, recommended treatment which relies on AmB administered for 14 days requires active monitoring and management of related side effects, not always possible in many settings. With existing animal data suggesting effectiveness of shorter courses AmB and small human studies evoking effectiveness oral combination of high dose fluconazole (1200 mg/day) and 5FC for inductive CM treatment, there was need for further exploration of these as possible alternatives for initial treatment of CM. Shorter course AmB would mean less side effects, oral therapy would mean few infusions and less constrains, all of which could be cheaper and sustainable. As such, a highly powered phase III, non-inferiority trial comparing oral fluconazole plus flucytosine or short course AmB (7 days) combination (fluconazole or 5FC) to standard AmB (14 days) combination (fluconazole or 5FC) was designed. This multicentre trial, spearheaded by a team at the St Georges’ University of London, in collaboration with the Institut Pasteur of Paris and London School of Hygiene and Tropical Medicine (LSHTM), was carried out in four countries in Africa, supported by the ANRS and the MRC. These four African centres included Cameroon, Malawi, Tanzania and Zambia.

Of note, in Cameroon, where there were two centres (Douala and Yaounde), it was the first randomised in-hospital phase III trial and a challenging one because of the severity of CM. More so, this was the first experience of large-scale use of intravenous AmB, the use of manometers for measuring opening pressure during LP and the use of CrAg tests and quantitative fungal colony count during CSF culture. This required reorganising patient management pathways as well training personnel on the diagnosis and standardised management of severely ill CM patients.

During the trial which lasted three years, 110 patients were included in the two centres in Cameroon for a total of 721 patients in all the centres. Initial results of the trial, presented as a late-breaker communication during the IAS conference in July 2017 in Paris showed that 7-

day AmB plus flucytosine or 14-day oral fluconazole plus flucytosine were non-inferior to standard 14-day AmB plus flucytosine in the initial management of CM. These findings in an African setting increases the options available to clinicians in the initial management of CM which could be tailored according to context and patient presentation.

In the context of systematic pre-ART CrAg screening and systematic LP to CrAg positive patients, patient in whom asymptomatic CM is confirmed (some of whom ambulatory) are most eligible for curative oral combination of high dose fluconazole plus flucytosine instead of standard pre-emptive fluconazole. More so, in a context of unavailable diagnostic CSF analysis or no systematic pre-ART screening (as is the present case of Cameroon), symptomatic patients with presumptive CM diagnosis could also be eligible for oral combination of fluconazole plus 5FC. Furthermore, in situations of standard diagnosis of CM, patients who are less severe with unaltered mental status are eligible for oral combination therapy and short course AmB plus 5FC reserved for patients in a coma or those with difficulties in swallowing. With these, in settings where AmB is available, we recommend short course (7-day) AmB plus 5FC instead of standard 14 days, as induction therapy for CM and if AmB is not available or monitoring of adverse events is not possible, oral high dose fluconazole plus 5FC should be the treatment of choice.

Furthermore, inductive treatment of CM as presently recommended relies on 14 days intravenous AmB plus 5FC whose use in many settings with high CM burden is limited by the lack of means of monitoring and treatment of AmB-related adverse events. During the multicentre ACTA trial, included patients were randomised into either an oral combination regimen or an AmB containing regimen (for either 7 or 14 days), adverse events were systematic monitored, pre-empted and treated. In Cameroon, where this was the first experience of large scale use of intravenous AmB for CM, we were interested in the incidence as well as factors associated to occurrence of severe adverse event during initial CM treatment. We therefore proposed to the ACTA trial scientific committee, a study on secondary analysis of aggregated trial safety data.

Preliminary results show active monitoring and treatment of adverse events resulted in overall fewer adverse event. Anaemia and kidney toxicities common with AmBd were decreased to about half of what is presently reported in other settings. This may be some evidence that active adverse event monitoring, a prerequisite for routine AmB use, is feasible in LMIC and results in better outcome.

Finally, in most studies in which patients are treated for CM, outcomes are reported during the first 10 weeks. CM a severe disease of the CNS has the potential of irreversible sequelae whose impact on patient survival goes beyond the mostly reported timeline of 10 weeks. In collaboration with Estelle Pasquier, as part of her MSc dissertation at the LSHTM co-supervised by Pr Olivier Lortholary, we carried out a systematic review on CM outcome beyond 3 months of treatment. The main findings show that little is reported on patient outcome beyond 10 weeks of treatment. Consequently, there were too few studies to do a meta-analysis. However, in HIV-related CM, four in five patients in HIC settings in the ART era were alive at one year while in LMIC, only 1 in 5 patients were alive. Among the factors associated with better long-term survival were induction treatment with AmB and aggressive management of raised ICP associated with CM.

With evidence of effectiveness of short course AmB plus 5FU and promises of its cost-effectiveness and sustainability in LMIC, iterating on systematic measurement of opening pressure during LP and management of high pressures by repeated subtractive LP are crucial for long term survival.

In addition to my own thesis work, I had the opportunity to be part of an advocacy manuscript to prioritise CM as a major public health problem. Indeed, HIV-associated CM, a primary cause of adult meningitis in LMIC is responsible for 15% of all-cause HIV-related mortality, second only to TB, thus worthy of global recognition and prioritisation as is the case with TB. With advances in HIV related medicine, such mortality is unacceptable high for a disease whose association to AIDs was discovered early-on, at the start of the HIV pandemic. Diagnosis of CM relies on simple and relatively affordable tools (CrAg detection, Indian ink stain and culture in sabouraud). Effective treatment of CM reposes only on three medications (AmB, fluconazole and 5FU) each of which are more than fifty years old and off-patent. Therefore, allocating resources specifically for providing simple diagnostic tools and treatment for CM in LMIC settings, plausibly will be more instrumental than ART alone, in decreasing HIV-related deaths, especially as patients failing ART are dying of CM. As such we suggested in an international collaborative write-up, that CM be recognised as a neglected tropical disease and major cause of adult mortality.

4. Perspectives

4.1. Contribution to local health policy on CM in Cameroon

Defining CM as a major public health problem in HIV-patients in Cameroon is crucial for pragmatic algorithmic strategies for optimal management to be put in place. The provision of simple and sustainable diagnostic tools as well advocacy for recommended treatment to be available and sustainable are primordial in decreasing morbidity and mortality. Training personnel on appropriate administration of AmB and the optimal management of raised intracranial pressure will markedly contribute to reduce all-cause HIV-related mortality. Creating opportunities for research to better understand and manage HIV-related CM

4.2. Avenue for further research

1. Further studies on the biobank obtained during the prospective study
 - We proposed a study on the molecular typing of clinical isolates of *Cryptococcus* spp. obtained from patients who participated in the Cameroon site of the ACTA trial with the aim of evaluating the impact of genotypes on early determinants of outcome. We obtained financial support for this study (ANRS 12367 MOTYCCIC) and recently obtained ethical clearance from the Cameroon national ethics committee for the study to be carried in the molecular mycology unit of Institut Pasteur.
 - We are designing other studies on the biobank of the ANRS 12312 PreCASA study to determine the prevalence of markers of other opportunistic infections
2. Pursue further research development training in clinical research
 - Aspiring application for the European and Developing Countries clinical trial partnership (EDCTP)-Africa Research excellence fund (AREF) career development fellowship

4.3. Career development plan

This piece of work is on the path of a career development plan which will comprise reference in patient care and clinical research in collaboration with the existent network of research institutions comprising Institut Pasteur of Paris, St Georges' university hospital London, Necker Hospital, the Cochrane team and others.

4.4. Experience gained

At the end of this challenging but enriching exercise, much experience was gained. The opportunity to conceive, design, implement and coordinate clinical studies in collaboration

with top and leading researchers in the field of cryptococcosis is an excellent template for an aspirant independent senior researcher. The chance to analyse data from a high quality well conducted controlled trial, a cohort study and carry out meta-analyses has greatly improved my clinical data management, analysis and interpretation skills. Other skills include communication, networking and collaboration with diversified experts.

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Summary of the thesis

Cryptococcal meningitis (CM), caused by an encapsulated yeast is a leading cause of AIDS related opportunistic infection in adults in sub-Saharan Africa and a major driver of mortality, second to tuberculosis. We aimed at optimising the management of AIDS-related cryptococcal meningitis in Cameroon through interventional studies. As such, we designed and performed three studies on the role of cryptococcal antigen (CrAg) in CM diagnosis, contributed in a major phase III non-inferiority clinical trial for inductive treatment of CM in the African setting and analysed the trial participants' tolerability of the antifungals used in the trial. We also contributed in a review on the long-term prognosis of CM and finally in an advocacy paper for CM to be recognised as a neglected tropical disease.

In Cameroon, serum CrAg detection, a major risk factor for incident CM in AIDS patient is prevalent in 7.5% of patients initiating antiretroviral therapy (ART) at less than 100 CD₄ cells/ μ L, of whom 45% have cerebrospinal fluid (CSF) evidence of asymptomatic CM. The new Biosynex CryptoPS test for CrAg detection is comparable to the IMMY lateral flow assay test and shows promise for correctly classifying patients with high serum CrAg titre, a predictor of confirmed CM. Post CrAg screening, enhanced adherence to ART and to fluconazole-based pre-emptive therapy to CrAg positive patients who present with no CM is effective in preventing incident CM within the first year of ART. In HIV patients presenting with symptoms of central nervous system disease, compared to Indian ink staining and/or culture of CSF, serum CrAg detection is highly presumptive of CM and CSF CrAg detection is diagnostic of first episode of CM. In African patients with confirmed CM, inductive therapy based on oral fluconazole-flucytosine combination or seven-day amphotericin B-flucytosine combination are as effective and more tolerated than standard fourteen-day amphotericin B-flucytosine combination. In spite advances in HIV care, mortality due to CM remains unacceptably high warranting CM to be recognised as a neglected tropical disease for which targeted resources need to be allocated to reduce HIV-related mortality.

Overall, in Cameroon, putting in place of local pragmatic algorithms based on the availability of simple but highly performant diagnostic tools and sustainable recommended treatment are indispensable to decrease AIDS-associated CM-related morbidity and mortality.