Title: High Cryptococcal Antigen Titers in Blood are Predictive of Subclinical Cryptococcal Meningitis Among HIV-Infected Patients

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Running title: Blood Cryptococcal Antigen Titers

Summary: Blood cryptococcal antigen (CrAg) titers are associated with concurrent subclinical cryptococcal meningitis in at least a third of CrAg-positive patients with advanced HIV, which may contribute to increased mortality. Blood CrAg titers can guide management in this population.

Abstract

Background

High mortality rates among asymptomatic cryptococcal antigen (CrAg)-positive patients identified through CrAg screening, despite pre-emptive fluconazole, may be due to undiagnosed cryptococcal meningitis.

Methods

Symptoms were reviewed in CrAg-positive patients identified through screening 19,233 individuals with CD4 cell counts < 100 cells/μL at 17 clinics and 3 hospitals in Johannesburg from September 2012 until September 2015, and 2 of these hospitals until June 2016. Cerebrospinal fluid from 90/254 (35%) asymptomatic patients and 78/173 (45%) with headache only was analyzed for cryptococcal meningitis, present if *Cryptococcus* was identified by India ink microscopy, culture, or CrAg test. CrAg titers were determined on stored blood samples from 62 of these patients. The associations between blood CrAg titer, concurrent cryptococcal meningitis, and mortality were assessed.

Results

Cryptococcal meningitis was confirmed in 34% (95% confidence interval (CI) 25%-43%, n=31/90) of asymptomatic CrAg-positive patients and 90%, (95% CI 81%-96%, n=70/78) with headache only.

Blood CrAg titer was significantly associated with concurrent cryptococcal meningitis in asymptomatic patients ($p<0.001$) and patients with headache only ($p=0.003$). The optimal titer for predicting cryptococcal meningitis was >160 (sensitivity 88.2%, specificity 82.1%); odds ratio for concurrent cryptococcal meningitis 34.5 (95% CI 8.3-143.1, $p<0.001$).

Conclusions

Around a third of asymptomatic CrAg-positive patients have concurrent cryptococcal meningitis. More effective clinical assessment strategies and antifungal regimens are required for CrAg-positive patients, including investigation for cryptococcal meningitis irrespective of symptoms. Where not possible to perform LPs on all CrAg-positive patients, blood CrAg titers should be used to target those most at risk of cryptococcal meningitis.

Keywords: Meningitis, Cryptococcal; Cryptococcosis; Diagnosis; Mass screening; Mortality

Background

Cryptococcal antigenemia is strongly predictive of subsequent cryptococcal meningitis in HIV-infected adults with CD4+ T-lymphocyte (CD4) counts of <100 cells/μL(1,2). This forms the basis of a 'screen-and-treat' approach to early detection, whereby HIV-infected patients with a CD4 count of <100 cells/μL are tested for cryptococcal antigen (CrAg) in blood (whole blood, plasma, or serum) prior to commencing antiretroviral therapy (ART). If the CrAg test is positive, and patients have no signs or symptoms of meningitis, they are treated with a pre-emptive course of fluconazole; 800 mg daily for two weeks, followed by 400 mg for eight weeks and then 200 mg pending immune reconstitution on ART. This strategy is included in World Health Organization (WHO) management guidelines for patients with advanced HIV (3–5), and adopted as recommended practice in several countries (6,7). However, the optimal management of patients who have cryptococcal antigenemia and do not have overt clinical evidence of meningitis is yet to be determined.

Recent evidence suggests that the CrAg screen-and-treat approach reduces the incidence of subsequent cryptococcal meningitis and death (8–10). However, most studies have found a persistent and independent association between cryptococcal antigenemia and mortality, despite pre-emptive fluconazole therapy,(8–12) implying that CrAg-positive patients may not be adequately investigated and treated under current guidelines.

A proportion of CrAg-positive patients may have meningeal infection with *Cryptococcus* without exhibiting any signs or symptoms, or complaining of a headache only(10,13). These cases of 'subclinical cryptococcal meningitis' are likely to be under-recognised, since WHO guidelines do not specifically recommend lumbar punctures (LPs) among asymptomatic CrAg-positive patients(4), and where they are routinely offered, LP uptake is poor(9,10,14). Furthermore,

many resource-limited settings where CrAg screening is now being implemented do not have access to the required equipment or health-workers with the ability to carry out LPs at the screening site(15).

Headache is a common and inconsistently reported complaint among patients with advanced HIV,(16–19) and prior studies have found having a headache to be a poor predictor of cryptococcal meningitis in CrAg-positive patients(10,19). Physicians therefore frequently omit to perform LPs if headache occurs without any other neurological signs or symptoms(9,10,14,17).

Studies have shown an association between blood CrAg titer and the development of subsequent cryptococcal meningitis and/or mortality in CrAg-positive patients(1,11,19,20). However, the relationship between blood CrAg titer and *concurrent* cryptococcal meningitis has not yet been systematically investigated. We performed a cross-sectional study to establish a) the prevalence of subclinical and minimally symptomatic (patients with headache only) cryptococcal meningitis, and b) whether blood CrAg titer was predictive of concurrent cryptococcal meningitis in CrAg-positive individuals identified during routine screening, who were asymptomatic or complained of headache only, and who had an LP performed. A subsequent prospective cohort study assessed the relationship between CrAg titre and concurrent cryptococcal meningitis, with mortality within six months.

Methods

The studies were conducted at 17 primary care clinics and 3 hospitals in Johannesburg from September 2012 until September 2015, and at 2 of these hospitals: Helen Joseph and Tambo

Memorial Hospital until June 2016. Ethics approval was granted by the University of the Witwatersrand and the London School of Hygiene and Tropical Medicine; the study protocol was also cleared by the Centers for Disease Control and Prevention. All HIV-infected individuals presenting to these facilities during the study period with a CD4 count of <100 cells/μL had a qualitative CrAg test performed in the laboratory using a lateral flow assay (LFA, ImmunoMycologics, Norman, Oklahoma, USA) on remnant ethylene-diamine-tetraacetic acid (EDTA)-containing blood from the CD4 count sample. If CrAg-positive, individuals aged >16 years were invited to participate in the study. If enrolled, information (including the presence of headache or confusion) was collected from them, or from their medical records, by professional study nurses using standardised structured questionnaires. CrAg-positive patients were managed by their usual health providers who received regular training on national guidelines for CrAg screening and treatment(6), delivered by study investigators. Recommended management was initial assessment for any symptoms or signs of meningitis and, if present, urgent referral for investigation of cryptococcal meningitis with LP and subsequent treatment as appropriate(6). If symptoms and signs of meningitis were absent, the guidelines suggested an LP should be considered 'if available'. If an LP was not performed, or if it excluded a diagnosis of cryptococcal meningitis, a course of pre-emptive fluconazole for at least 12 months was recommended as per WHO guidance(4).

We carried out a cross-sectional study to establish the prevalence (using exact binomial confidence intervals) of concurrent cryptococcal meningitis, and the relationship with blood CrAg titer, in participants with neither headache nor confusion, and those with headache only, who had an LP performed within a month of review of their CrAg test result. A sample size of 88 and 62 participants was required to determine an estimated prevalence of concurrent cryptococcal meningitis of 35% and 80% in the asymptomatic and headache only groups respectively, with 10% precision. Concurrent cryptococcal meningitis was defined as occurring

in those who had *Cryptococcus* identified by cerebrospinal fluid (CSF) microscopy with India ink, fungal culture, and/or CSF CrAg testing.

CrAg-positive whole blood samples were sent from the diagnostic facilities to the reference laboratory during the study period, and were stored at -70˚C. CrAg titer was determined using the CrAg LFA on serially-diluted samples of thawed unspun whole blood, using manufacturer's instructions. Titers were read manually by three investigators, who were blinded to the CSF results and to each others' readings. Serially-diluted blood samples were tested until the next reading was negative. If discordant, the higher reading was used as long as there was agreement within a double dilution.

The association between blood CrAg titer and concurrent cryptococcal meningitis was tested using a Mann-Whitney U test for the 'asymptomatic', the 'headache only' and combined groups. A receiver operating characteristic curve was used to establish an optimal 'cut-off' titer that could be used to screen for concurrent cryptococcal meningitis, and the sensitivity and specificity of this titer was determined. The cut-off titer was then used to estimate the odds ratio of concurrent cryptococcal meningitis with a high blood CrAg titer and other variables were assessed for their association with cryptococcal meningitis.

Prospective data on ART, antifungal treatment and mortality were obtained from clinic and phone-call follow-up and/or review of clinical and laboratory records for up to three years. Progression to death within six months in those with/without concurrent cryptococcal meningitis and with high/low blood CrAg titer was examined by Kaplan-Meier estimates and a multivariate Cox proportional hazards model. The following variables were considered

potential confounders: age, sex, baseline CD4 count, ART status, headache and whether or not patients received any antifungal therapy following LP. Assuming a 15% risk of death among those with a blood CrAg titer ≤160 (based on a previous study(20)), with a two-sided significance of 95% and power of 80%, a sample size of at least 120 individuals was required in each group to detect at least a 15% difference in mortality among those with blood CrAg titer >160.

Results

Of 19,233 HIV-infected patients over the age of 16 years with CD4 counts <100 cells/μL who were screened during the study period, 851 were CrAg-positive (4.4%, 95% CI 4.1% - 4.7%). Demographic and clinical data (Supplementary Table 1) including signs and symptoms of meningitis were available for 505 (59.3%) patients. Of these, 254 (50.3%, 95% CI 45.8 - 54.7%) reported no headache or confusion at the time of CrAg test, 173 (34.3%, 95% CI 30.1 - 38.6%) complained of a headache without any confusion, and 78 (15.4%, 95% CI 12.4% - 18.9%) were confused (see Figure 1). There were no significant differences in age (p=0.2), sex (p=0.5) or CD4 cell count (p=0.3) among those groups with no symptoms, headache only or confusion (Supplementary Table 1).

CSF results from an LP performed within 30 days of receiving their CrAg result (median 2.5 days, interquartile range (IQR) 1-7) were available for 90/254 (35.4%) of asymptomatic CrAg-positive patients and 78/173 (45.1%) of patients with headache only. Of note, no LP was recorded for 50/173 (28.9%) of patients who complained of a headache (and results were not available for the other 45 patients who were recorded to have had an LP). There was no significant difference in age or sex; however, CD4 count was lower in those who had an LP

performed compared to those who did not (median 22 cells/μL, IQR 7 – 39 vs. 27 cells/μL, IQR 10-53, p=0.01) (Supplementary Table 1).

On analysis of CSF, 31/90 (34%, 95% CI 25% - 45%) asymptomatic patients and 70/78 (90%, 95% CI 81% - 96%) patients with headache only had evidence of meningeal infection with *Cryptococcus.* Having a headache compared with no symptoms was strongly predictive of concurrent cryptococcal meningitis (OR 16.7, 95% CI 7.1 – 39.0, p<0.001). There was no significant difference between patients with and without concurrent cryptococcal meningitis in terms of age (median, 37 vs. 39 years, p=0.24) or sex (male, 53% vs. 44%, p=0.3) although CD4 counts were lower in those with concurrent cryptococcal meningitis (median 19 (IQR 5-35) vs. 25 (10-42) cells/μl, p=0.06).

Thirty-seven (41%) of the 90 asymptomatic patients with CSF results, and 25 (32%) of the 78 with headache only had stored samples available for blood CrAg titer analysis. Patients from whom blood samples were available were not significantly different to those without in terms of age, sex or CD4 count ($p \geq 0.05$) (Supplementary Table 1). Titers ranged from <5 to 2560 (median 40, IQR 10-160) in those with no evidence of meningeal involvement, and 40 to 6.7×10^7 (median 5120, IQR 1280-81920) in those with cryptococcal meningitis (Figure 2). Blood CrAg titer was significantly associated with cryptococcal meningitis in both asymptomatic patients ($p<0.001$) and patients with headache only ($p=0.003$) with area under the receiver operating characteristic curve of 0.93 (Figure 3). The optimal cut-off titer for predicting concurrent subclinical cryptococcal meningitis was >160, with sensitivity of 88.2% and specificity 82.1% (Table 1). A blood CrAg titer of >160 had an odds ratio of 34.5 for concurrent cryptococcal meningitis (95% CI 8.3-143.1, $p<0.001$) in the combined group and 11.2 (95% CI 2.3 - 54.6, $p=0.002$) in the asymptomatic group.

The association between a blood titer of >160 and concurrent cryptococcal meningitis remained significant, even when adjusted for CD4 count (OR 38.4, 95% CI 8.0 – 185.0, $p<0.001$).

All participants with symptom review and LP results available (n=168) were followed up for a median of 37 days (range 1-180 days). Of those with available data, 89/107 (83%) were started on appropriate antifungal therapy; 44/45 (98%) of patients with cryptococcal meningitis received intravenous amphotericin B and oral fluconazole and 45/62 (73%) of those without received oral fluconazole.

Of 101 patients who had concurrent cryptococcal meningitis, 22 (22%) died and 6 (6%) were lost to follow up during the first 6 months, compared to 12 (18%) and 8 (12%) of 67 patients without cryptococcal meningitis. There was no association found between age, sex, CD4 count, CrAg titer, headache or ART status, and risk of death. However, receipt of any antifungal agent (amphotericin B, fluconazole or a combination) was found to be protective (HR 0.20, 95% CI 0.08 – 0.49, p<0.001). When adjusting for receipt of antifungal therapy, the HR for death in those with concurrent cryptococcal meningitis was 2.00 (95% CI 0.83-4.78, p=0.12) in asymptomatic patients (Figure 4) and 1.82 (95% CI 0.88-3.79, p=0.11) in the combined cohort. There remained no significant association between CrAg titer and risk of mortality (HR 1.58, 95% CI 0.57 – 4.36, p=0.38).

Discussion

More than a third of CrAg-positive patients with no signs or symptoms of meningitis, and 90% of those complaining of a headache only, had evidence of meningeal involvement on CSF analysis. In both groups, higher blood CrAg titers were associated with an increased risk of concurrent cryptococcal meningitis. A cut-off of >160 had moderate sensitivity (88.2%) and specificity (82.1%) for predicting CNS disease; however, no single titer cut-off could distinguish between those with and without concurrent cryptococcal meningitis with 100% accuracy.

Previous studies have found higher CrAg titers to be associated with subsequent cryptococcal meningitis and mortality with a titer of around >160 consistently indicating increased risk (Table 2). Apart from our study, limited evidence exists for an association between CrAg titer and concurrent cryptococcal meningitis in patients *without* symptoms or signs of meningitis, or complaining of a headache only. Where CrAg-positive patients have received LPs following screening, the prevalence of concurrent cryptococcal meningitis is 25%-78%, with increased

risk in those with higher blood CrAg titers(10,13,19,21). However, many of these studies include symptomatic as well as asymptomatic patients. Our study established a significant risk of subclinical concurrent cryptococcal meningitis, which was associated with higher blood CrAg titers. This finding is important for informing management of CrAg-positive patients identified through the expansion of screening programmes worldwide.

In our study, LPs were offered at the discretion of patients' health practitioners, who may have elicited different signs and symptoms from patients than those recorded by study nurses. This may have led to an overestimation of the burden of concurrent cryptococcal meningitis, and a stronger association with headache than has been previously observed, since LPs would have been more likely offered to patients for whom there was a greater degree of suspicion of cryptococcal meningitis, and for patients admitted to health facilities where LPs were readily available. Patients who received LPs had lower CD4 counts than those who did not (22 vs. 27 cells/μL). Of note, the proportion of patients with subclinical cryptococcal meningitis observed was similar to proportions reported in previous studies(10,13).

Our study was also limited by the number of patients with available data, which did not reach the required sample size for survival analysis and may explain the lack of significant association between concurrent cryptococcal meningitis or high CrAg titer and mortality, as previously described(1,17,20). Additionally, almost all patients who were diagnosed with cryptococcal meningitis and had available antifungal data received some amphotericin B, which may have reduced mortality in this group and those with a high blood CrAg titer. Other variables which are known to affect prognosis, but which were not available for analysis in this study, include the presence of cryptococcal immune reconstitution inflammatory syndrome (IRIS), adherence

to antifungal therapy, concomitant diseases, HIV viral load, CSF cell counts and LP opening pressure, as well as whether or not repeated LPs were performed.

Despite these limitations, the results highlight a substantial risk of concurrent cryptococcal meningitis among patients who are found to be CrAg-positive on screening but lack symptoms and signs that typically lead clinicians to investigate for meningitis with an LP. Since patients with cryptococcal antigenemia, treated pre-emptively with fluconazole therapy, have a 2.5-fold increased risk of death when compared to individuals with similar CD4 counts without cryptococcal antigenemia(8–11), a more aggressive approach to management should be considered. Where performing LPs is possible, these should be used routinely to investigate for cryptococcal meningitis in all CrAg-positive patients, even those without symptoms. However, experience suggests that only a minority of asymptomatic or mildly symptomatic patients are willing to undergo LP, even if carefully counselled. In this study, more than a quarter of CrAg-positive patients with a headache did not have an LP performed, although concurrent cryptococcal meningitis was diagnosed in 90% of those who did. Alternatively, blood CrAg titer could be used to tailor patient management. Those with higher CrAg titres, perhaps identified with a semi-quantitative CrAg test, could be targeted for more intensive, but still feasible and sustainable, antifungal therapy. Further work is required to determine whether such an approach could improve the outcomes of patients with cryptococcal antigenemia.

Acknowledgements

Assistance with data retrieval (National Institute for Communicable Diseases): Phelly Matlapeng; Saneliswe Nkabinde; Matshediso Mkhwanazi; Siphiwe Kuta; Neo Legare

Disclaimer:

The findings and conclusions in this paper are those of the authors and do not necessarily represent the official positions of their sponsoring agencies.

Funding

This work was supported by the South African Medical Research Council (self-initiated research grant awarded to T.O), the Meningitis Research Foundation (1604.0 awarded to R.W.) and the U.S. Centers for Disease Control and Prevention (CDC-RFA-GH15-1575 awarded to the National Health Laboratory Service, principal investigator N.P.G.)

Potential Conflicts of interest

Rachel M Wake: No conflict

Erika Britz: No conflict

Charlotte Sriruttan: No conflict

Ivy Rukasha: No conflict

Tanvier Omar: No conflict

David C Spencer: No conflict

Jeremy S Nel: Received speaker's fee for lecture on disseminated cryptococcosis from Mylan N.V.

Sello Mashamaite: No conflict

Adeboye Adelekan: No conflict

Tom M Chiller: No conflict

Joseph N Jarvis: No conflict

Thomas S Harrison: Received consultancy fee from Viamet Pharmaceuticals Inc., honoraria from Pfizer Inc., and tests for research purposes from Immuno-Mycologics

Nelesh P Govender: No conflict

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Table 1 Sensitivity and specificity at blood CrAg titer cut-off levels, tested by serial dilution method, for concurrent cryptococcal meningitis among patients with no symptoms or signs of meningitis and those complaining of a headache only (n=62)

Blood CrAg titer cut-off for predicting concurrent cryptococcal meningitis	**Sensitivity (%, 95% CI)**	**Specificity (%, 95% CI)**
>5	100 (89.7-100)	21.4 (8.3-41.0)
>10	100 (89.7-100)	32.1 (15.8-52.4)
>20	100 (89.7-100)	46.4 (27.5-66.1)
>40	97.1 (84.7-99.9)	57.1 (37.2-75.5)
>80	91.2 (76.3-98.1)	71.4 (51.3-86.8)
>160	88.2 (72.5-96.7)	82.1 (63.1-93.9)
>320	79.4 (62.1-91.3)	85.7 (67.3-96.0)
>640	76.5 (58.8-89.3)	89.2 (71.8-97.7)
>1280	70.6 (52.5-84.9)	96.4 (81.7-99.9)
>2560	61.8 (43.6-77.8)	100 (87.7-100)

Table 2 Studies including an analysis of blood CrAg titer with cryptococcal meningitis and/or mortality

Country	**Year**	**Study design**	**Association between blood CrAg titer and cryptococcal meningitis and/or mortality**	**Notes**	**Ref**
Association between blood CrAg titer and *subsequent* cryptococcal meningitis and/or mortality					
South Africa	2009	Retrospective analysis of CrAg titers (using Latex Agglutination, LA) on pre-ART blood samples from 46 patients.	Higher baseline titer associated with increased risk of mortality (p=0.02), subsequent cryptococcal meningitis (p=0.03) and relapse (all >512) within 1 year.	No baseline LP performed. Symptoms unknown.	(1)
Tanzania	2015	Retrospective analysis of blood CrAg titers (using LFA) on pre-ART blood samples from 21 asymptomatic patients.	Titer of >160 associated with subsequent cryptococcal meningitis (adjusted OR, 4.83; 95% CI, 1.24–8.41; *P* = .008) within 1 year.	3 patients with titers ≤160 died of unknown causes.	(11)
Uganda	2016	Cluster randomized trial of CrAg screen and treat strategy. CrAg titers (LFA) on 151 asymptomatic patients.	Titer of ≥160 associated with subsequent cryptococcal meningitis (HR OR 9.2, 95% CI 2.14-39.58, p<0.01) (unpublished data)	Increased risk of death/subsequent cryptococcal meningitis if titer ≥160 and CD4 ≤50.	(20)
Tanzania	2011	CrAg screening of all HIV positive hospital admissions. Serum CrAg titers (LFA) on 17/333 CrAg-positive patients.	Higher titer associated with mortality (p=0.004).	All symptomatic, 15/17 had cryptococcal meningitis.	(17)

Association between blood CrAg titer and *concurrent* cryptococcal meningitis					
Democratic Republic of Congo (DRC)	1989	Cross-sectional study of 44 newly diagnosed HIV infected adults performing baseline blood and CSF CrAg titers (LA).	Higher titers associated with increasing risk of concurrent cryptococcal meningitis (PPV 92% for titer >128).	Concurrent cryptococcal meningitis present in 29/44 (66%) CrAg-positive patients. Symptoms unknown.	(21)
South Africa	2016	Prospective study implementing CrAg screen and treat. Blood CrAg titers (LFA) on 10 patients who had a baseline LP.	Higher titers associated with concurrent cryptococcal meningitis No cryptococcal meningitis in patients with titers of <160.	Concurrent cryptococcal meningitis present in 4/10 (40%). Symptoms unknown.	(10)
Cambodia	2007	Cross-sectional study screening patients with CD4 ≤200 cells/μL and performing LPs in all CrAg-positive patients (n=53).	Median titer (LA) higher in those with than those without concurrent cryptococcal meningitis (2048 vs. 16, p<0.0001).	Concurrent cryptococcal meningitis present in 41/53 (78%). Most symptomatic.	(19)
Thailand	2010	Retrospective study performed blood CrAg titers (LA) on 12 asymptomatic CrAg-positive patients who had baseline LPs.	Higher CrAg titers in those with cryptococcal meningitis than those without (128-1024 vs. 8-128).	Concurrent subclinical cryptococcal meningitis in 3/12 (25%).	(13)

Figure Legends

Figure 1 Flow-chart to show individuals included and excluded from studies

Figure 1 Blood CrAg titres in A) asymptomatic CrAg-positive patients (n=37) B) minimally symptomatic CrAg-positive patients (n=25) and C) combined cohort of asymptomatic and minimally symptomatic patients (n=62) with and without concurrent cryptococcal meningitis

Figure 2 Receiver operating characteristic curve for blood CrAg titre and cryptococcal meningitis among patients with no symptoms or signs of meningitis and those complaining of a headache only (n=62)

Figure 3 Kaplan-Meier survival estimates in asymptomatic CrAg-positive patients, n=90 with subclinical cryptococcal meningitis and without subclinical cryptococcal meningitis. Abbreviations: HR, hazard ratio; cmcat, cryptococcal meningitis category; pos, positive; neg, negative.

Figure 1

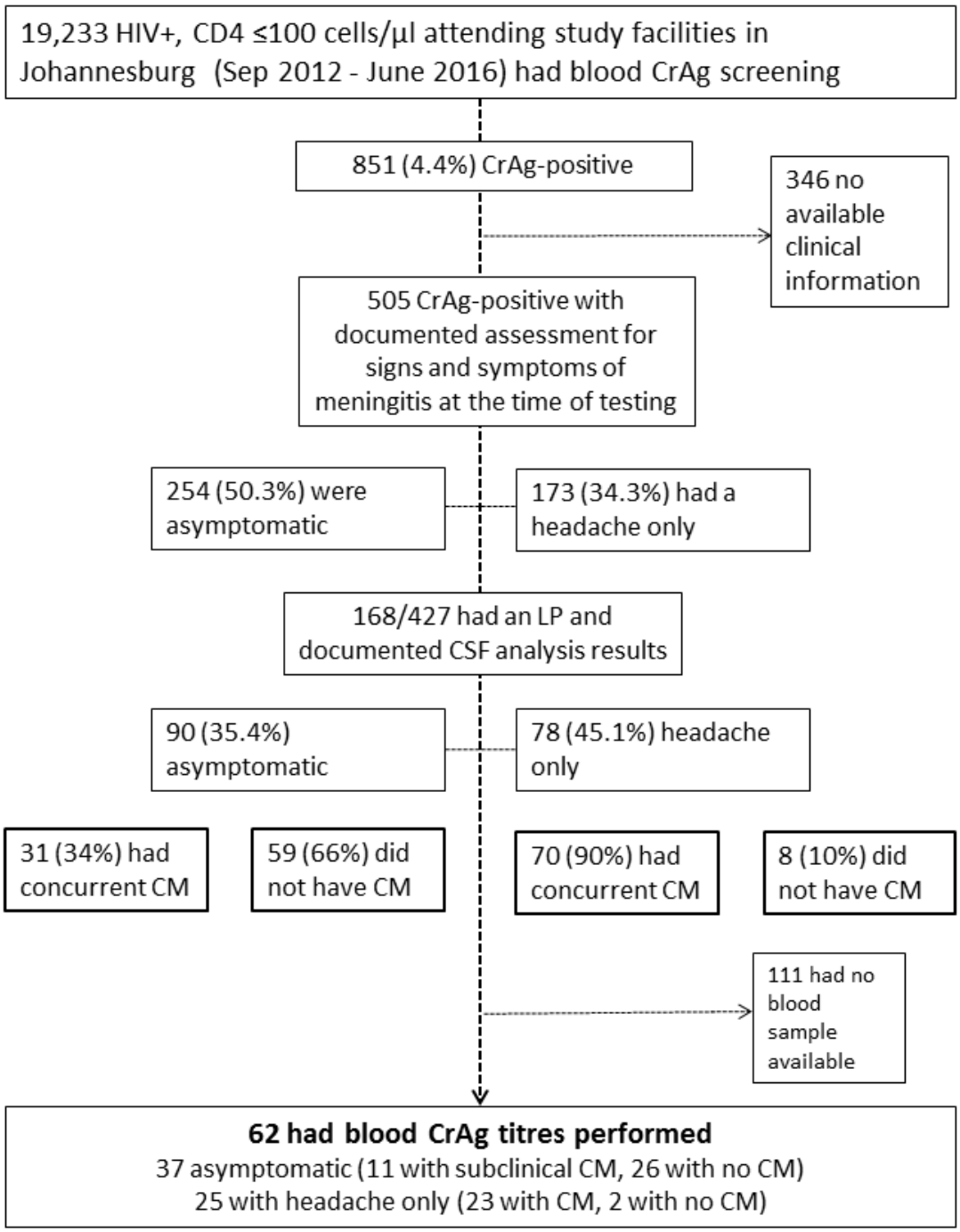

Figure 2

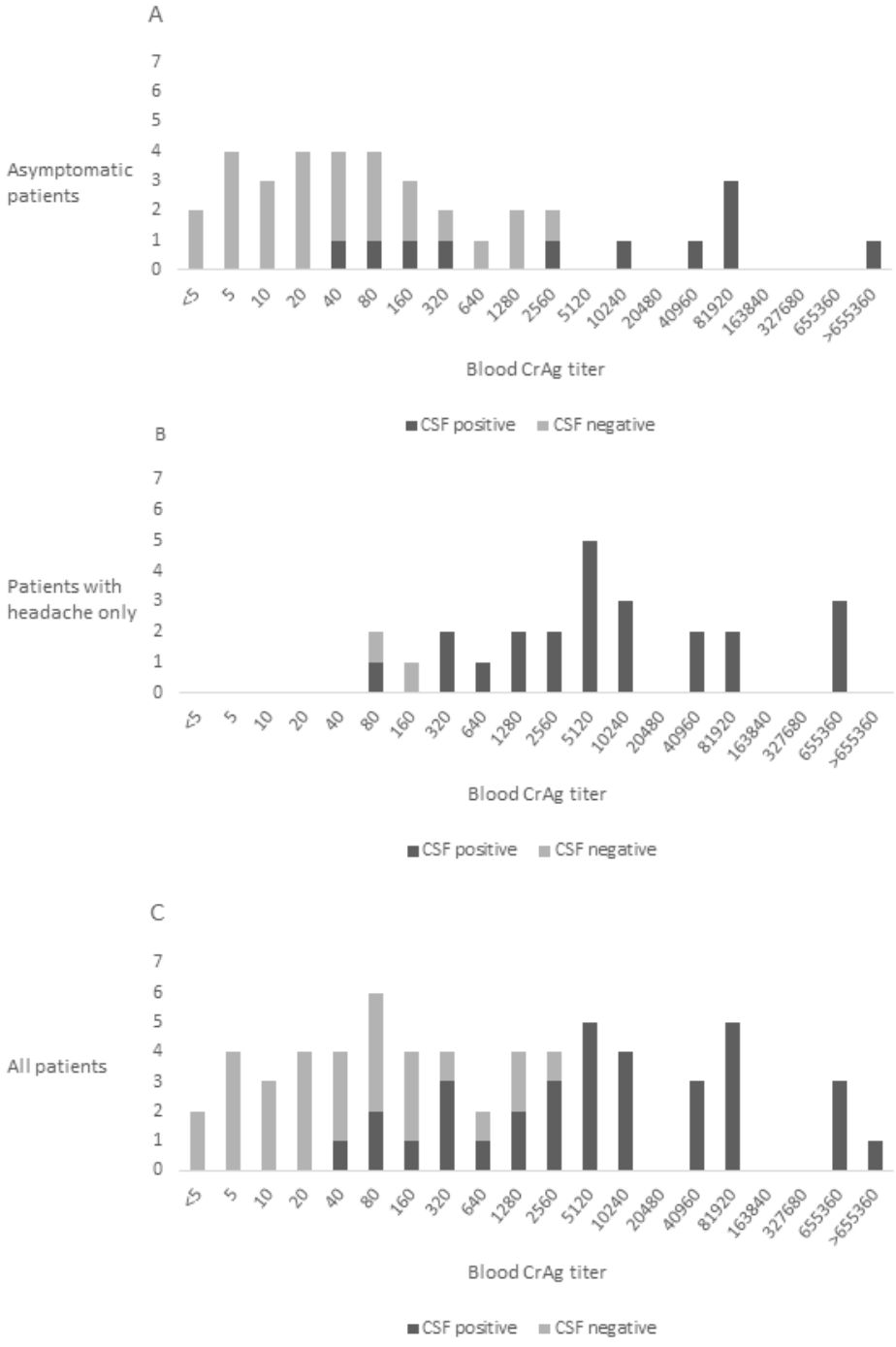

Figure 3

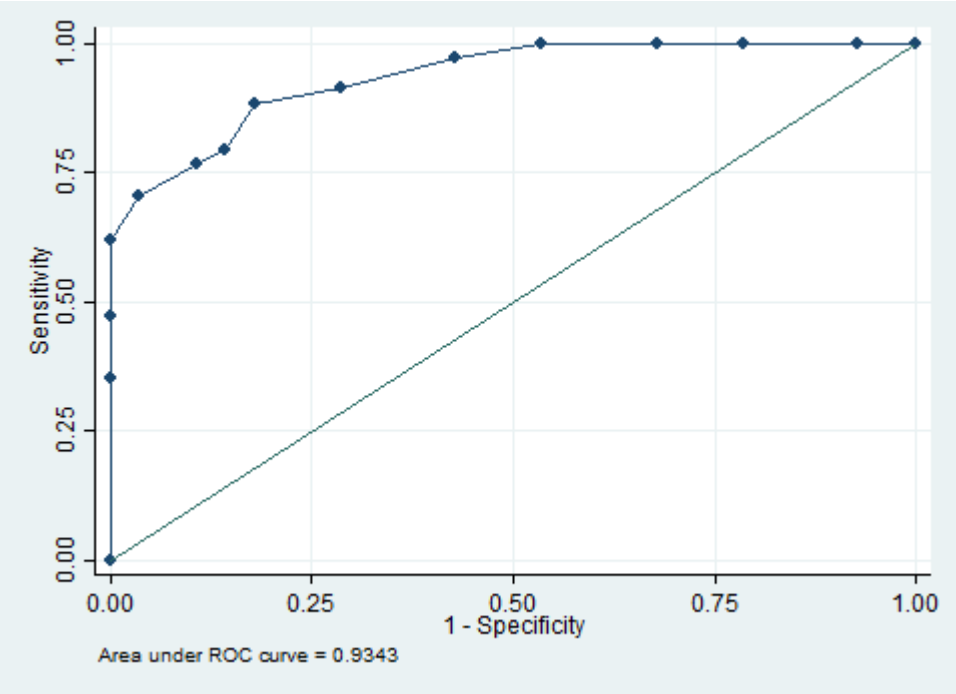

Figure 4