Evaluation of a Novel Semi-quantitative Cryptococcal Antigen Lateral Flow Assay in Patients with Advanced HIV Disease

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Short title: IMMY CrAgSQ Evaluation

Short summary: The novel semi-quantitative CrAgSQ cryptococcal antigen (CrAg) test had high sensitivity and specificity compared to current CrAg lateral flow assay and provided quantitative CrAg results which were associated with both CrAg titers derived from dilutional testing and clinical outcomes.

Key words: Cryptococcal meningitis, cryptococcal antigen, HIV, validation study, diagnostic accuracy; lateral flow assay

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ABSTRACT

Background: Higher cryptococcal antigen (CrAg) titers are strongly associated with mortality risk in individuals with HIV-associated cryptococcal disease. Rapid tests to quantify CrAg level may provide important prognostic information and enable treatment stratification.

Methods: We performed a laboratory-based validation of the semi-quantitative IMMY CrAgSQ assay against the current gold-standard CrAg tests. We assessed diagnostic accuracy of the CrAgSQ in HIV-positive individuals undergoing CrAg screening; determined the relationship between CrAgSQ scores and dilutional CrAg titers; assessed inter-rater reliability; and determined clinical correlates of CrAgSQ scores.

Results: A total of 872 plasma samples were tested using both CrAgSQ and conventional IMMY CrAg LFA tests; 692 sequential samples from HIV-positive individuals undergoing CrAg screening and an additional 180 known CrAg-positive plasma samples archived from prior studies. Inter-rater agreement in CrAgSQ reading was excellent (98.17% agreement, Cohen's Kappa 0.962, $p<0.001$). Using IMMY LFA as a reference standard, CrAgSQ was 93.0% sensitive (95% confidence interval [CI] 80.9%-98.5%) and 93.8% specific (95%CI 91.7%-95.6%). After reclassification of discordant results using CrAg enzyme immunoassay testing, sensitivity was 98.1% (95%CI 90.1%-100%), and specificity 95.8% (95%CI 99.1%-100%). Median CrAg titers were 1:10 (IQR 1:5-1:20) in the CrAgSQ1+ category; 1:40 (IQR 1:20-1:80) in the CrAgSQ2+ category; 1:640 (IQR 1:160-1:2560) in the CrAgSQ3+ category; and 1:5120 (IQR 1:2560-1:30720) in the CrAgSQ4+ category. Increasing CrAgSQ scores were strongly associated with 10-week mortality.

67 *Conclusions:* The CrAgSQ test had high sensitivity and specificity compared to the IMMY CrAg LFA test
68 and provided CrAg scores associated with both conventional CrAg titers and clinical outcomes.
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INTRODUCTION

Cryptococcal meningitis is a major cause of morbidity and mortality among people living with HIV (PLWH), resulting in an estimated 15% of HIV-related deaths worldwide (1). The incidence of HIV-associated cryptococcal meningitis has remained high in many low and middle-income settings despite improved population-level access to antiretroviral therapy (ART) (1-3). Mortality rates from cryptococcal meningitis with currently available treatments also remain unacceptably high, ranging from 25%-45% at ten weeks (4-7). There is an urgent need to improve both prevention and treatment strategies (8, 9).

Detection of the cryptococcal capsular polysaccharide antigen glucuronoxylomannan (GXM), commonly known as cryptococcal antigen (CrAg), in body fluids including cerebrospinal fluid (CSF) and blood (whole blood, plasma, or serum) is the cornerstone of diagnosis. Highly sensitive and specific CrAg lateral flow assays (LFAs) (10, 11), appropriate for use in laboratories with limited facilities or at the point of care (12), have markedly facilitated rapid clinical diagnosis of cryptococcal meningitis, and also enabled the implementation of CrAg screening programs aimed at detecting and treating early asymptomatic infection in HIV-positive individuals with low CD4 T-cell counts (13-16). In addition to providing a qualitative (positive/negative) result, higher CrAg titers have been shown to be strongly associated with increased fungal burden and mortality risk in patients with cryptococcal meningitis (17), and with the presence of central nervous system (CNS) infection and mortality risk among asymptomatic CrAg-positive individuals identified through (blood/plasma) CrAg screening programs (2, 18, 19). Determining CrAg titers may therefore provide important prognostic information, enabling stratification of treatment in individuals with cryptococcal meningitis, and identification of the CrAg-positive individuals detected through CrAg screening programs who require lumbar puncture (LP) to rule out CNS infection or may benefit from more intensive antifungal therapy.

Currently-used commercial CrAg assays provide a qualitative dichotomous positive/negative result, necessitating the testing of serial dilutions of the primary specimen to obtain a quantitative titer result. This serial testing involves significant additional costs, is time-intensive, and requires laboratory operator expertise which limits performance in clinical practice, and therefore it is rarely performed in low resource settings. To overcome these limitations, IMMY (Norman, OK, USA) has developed a new immunochromatographic test system for the semi-quantitative detection of the capsular polysaccharide antigens of the *Cryptococcus neoformans/gattii* species complex in serum, plasma, whole blood, and CSF (the "CrAgSQ" LFA) (Figure 1). We performed a comprehensive laboratory-based validation study to evaluate the performance of the CrAgSQ assay against the current gold-standard CrAg detection tests, assessing the diagnostic accuracy of the CrAgSQ assay in HIV-positive individuals undergoing CrAg screening; determining the relationship of CrAgSQ band cut-offs with conventionally-derived CrAg titers; assessing inter-rater reliability to inform interpretability of the CrAgSQ assay; and determining clinical correlates of CrAgSQ results.

METHODS

Study Population and procedures.

The study was performed in two parts (Figure 2). In the first part, the qualitative (positive / negative) performance of the CrAgSQ assay was evaluated against the first-generation qualitative IMMY CrAg LFA in a consecutively-recruited cohort of HIV-positive individuals with CD4 cell counts ≤200 cells/µL undergoing reflex CrAg screening at the Botswana-Harvard HIV Reference Laboratory (BHHRL) in Gaborone, Botswana, between January and August 2018 ("cohort 1"). BHHRL performs almost all CD4 testing for 27 public ART clinics and a national referral hospital in greater Gaborone. Residual EDTA whole blood sent to the BHHRL for routine CD4 testing found to have a CD4 ≤200 cells/µL underwent

CrAg screening using the IMMY CrAg LFA as part of a reflex CrAg screening study. The IMMY CrAg LFA is an immunochromatographic CrAg test that provides a qualitative test result within 15 minutes. The assay has been extensively validated on whole blood, serum, plasma, and CSF samples and is highly-accurate compared to other commercial assay types such as available enzyme immunoassays (EIAs) (10, 11). Plasma was separated and stored in -80°C freezers at the Botswana National Health Laboratory for subsequent CrAgSQ testing and IMMY CrAg LFA titer determination on completion of study recruitment. The sensitivity and specificity of the CrAgSQ were determined against the IMMY CrAg LFA as a reference standard, with all positive CrAgSQ scores (1+, 2+, 3+, 4+, or 5+) considered positive.

The second part of the study evaluated the relationship between CrAgSQ semi-quantitative results (CrAgSQ "score") and (a) IMMY CrAg LFA titers, and (b) clinical outcome (CNS disease in the CrAg screened population, and 10-week mortality in all cases). These assessments were performed using the plasma samples from cohort 1 described above, plus a collection of stored frozen plasma samples that had previously tested CrAg-positive using the IMMY CrAg LFA ("cohort 2"). Cohort 2 consisted of all plasma samples with positive CrAg tests from a 2015-2016 CrAg screening cohort study performed at BHHRL (2, 20) and from a phase II randomized-controlled trial (RCT) evaluating cryptococcal meningitis therapies in Gaborone, Botswana (21).

All CrAgSQ assays were performed by trained laboratory technicians according to manufacturer's instructions and read by two independent laboratory technicians blinded to previous CrAg test results as well as the other technician's read. Results were recorded as negative or 1+, 2+, 3+, 4+, or 5+ (Figure 1). The CrAgSQ test turn around time is approximately 15 minutes, similar to the conventional IMMY LFA test, and relies on a visual reading of line color intensity thus is subject to a degree of operator dependency. Discordant reads between technicians were arbitrated by a third investigator and a photographic record of all test strips maintained (see supplementary materials). Samples with discrepant qualitative results from the IMMY LFA and CrAgSQ assays, i.e. one positive and the other

negative, were re-tested at an independent accredited laboratory using a commercial enzyme immunoassay (EIA) [IMMY, Norman, OK, USA] by a scientist blinded to previous results.

Patients identified as CrAg-positive during the CrAg screening studies were treated according to an algorithm based on World Health Organization (WHO) guidelines [9,11], recommending high-dose fluconazole (1200mg/day) for asymptomatic individuals, LP to rule out CNS infection, and referral for amphotericin B-based treatment as an inpatient if CSF CrAg-positive (supplementary material figure s1). In the 2015-16 screening study, management decisions were at the discretion of the patients' healthcare providers, and the research team had no direct patient contact, thus were unable to directly assess adherence to treatment guidelines. In the 2018 screening study the research team actively managed CrAg-positive patients. Patient follow-up data were collected to six-months. The phase II RCT has been described in detail elsewhere (21). Participants were treated with either amphotericin B deoxycholate or liposomal amphotericin B, both given with fluconazole, as inpatients, and actively followed-up to ten weeks.

The research was approved by Institutional Review Boards at the University of Botswana, the Botswana Ministry of Health and Wellness, and the University of Pennsylvania. As the two CrAg screening studies were limited to implementation of a laboratory-based, WHO-endorsed screening intervention, and collection of routine clinical and outcomes data, a waiver of informed patient consent was granted. All patients in the phase II treatment trial provided written informed consent providing permission for sample storage and testing.

Validation study analysis

Sensitivity and specificity: Using cohort 1, we calculated the sensitivity, specificity, positive and negative predictive values of the CrAgSQ assay for qualitative (positive/negative) CrAg detection in plasma using the first-generation IMMY LFA test as the reference standard. In order to better classify potential false-

positive and false-negative results, an additional analysis was performed in which the IMMY EIA test result was used as a "tie-breaker" for samples with discrepant test results between the CrAgSQ assay and IMMY CrAg LFA, and the reference test result reclassified accordingly. Sensitivity, specificity, positive and negative predictive values were recalculated against this tie-breaker adjusted composite reference standard.

Inter-rater reliability: We assessed inter-rater reliability for the CrAgSQ assay using all samples from cohort 1 and cohort 2. Percent agreement between the two reading technicians was determined using an unadjusted Cohen's Kappa statistic. As the CrAgSQ test has ordered categorical values we calculated a second weighted Kappa with 75% weight given to values that were within 1 category between the two reviewers, (e.g. readings of 3+ and 4+), 50% weight given to values within 2 categories, 25% weight within 3 categories, and 0% weighting for a discrepancy of four categories (22).

Association between CrAgSQ quantification and CrAg titers: To evaluate the associations between semi-quantitative CrAgSQ results and CrAg titer values, median CrAg titers were calculated in each CrAgSQ result category using all samples form cohorts 1 and 2, and the results displayed graphically.

Relationship between CrAgSQ results and clinical outcomes: The proportion of CrAg-screened individuals with confirmed CNS infection (cryptococcal meningitis) at baseline in cohort 1 and 2 (excluding those in the phase II treatment trial), and the overall proportion of individuals who died by ten weeks were calculated according to plasma CrAgSQ category, and the association between plasma CrAgSQ result and ten-week mortality examined using a Cox proportional hazards model. A sensitivity analysis was performed in which all individuals lost to follow-up were assumed to have died.

All analyses were performed using STATA version 14 (Stata Corporation, College station, TX). P-values of <0.05 were considered significant.

RESULTS

A total of 872 plasma samples were tested using both the CrAgSQ and IMMY CrAg LFA tests; 692 from cohort 1 and 180 from cohort 2 (Figure 2). Baseline characteristics of the study participants are shown in Table 1. Inter-rater agreement in CrAgSQ reading was excellent, with 98.2% agreement, Cohen's Kappa 0.96, p<0.001 (Table 2A and 2B).

Of the 692 samples tested in the sequentially-enrolled cohort 1, 43 (6.2%) were positive for CrAg using the IMMY CrAg LFA. Compared to the IMMY CrAg LFA as a reference standard, CrAgSQ (used as a qualitative test) was 93.0% sensitive (95% confidence interval [CI] 80.9% – 98.5%) and 93.8% specific (95% CI 91.7% – 95.6%) (Table 2D). Forty of the 649 (6.2%) IMMY CrAg LFA negative samples were positive on CrAgSQ testing, all at the lowest 1+ score, and classified as false-positive; 3 of the IMMY CrAg LFA positive samples (all with the lowest titer of 1:2) were negative on CrAgSQ testing, and classified as false-negative.

On EIA testing, 13 of the 40 CrAgSQ "false-positive" samples were found to be CrAg positive with EIA readings above the optical density cut-off of 0.265 as specified by the manufacturer, thus reclassified as true-positives; and 2 of the 3 CrAgSQ "false-negatives" were negative on EIA testing, thus reclassified as true-negatives (Figure 3). Following this adjustment to the reference standard, the sensitivity of the CrAgSQ was 98.1% (95% CI 90.1 – 100%), and the specificity was 95.8% (95% CI 99.1% - 100%); 27 (4.2%) of IMMY CrAg LFA|EIA negative samples were CrAgSQ positive, all at the lowest 1+ score, and classified as false-positive; 1 of the IMMY CrAg LFA|EIA positive samples (at the lowest titer of 1:2) was negative on CrAgSQ testing, and classified as false-negative. Detailed clinical information for these unreconciled discordant results (27 false-positives and 1 false-negative) are shown in supplementary table 1.

Combining all 223 CrAg-positive plasma samples from cohort 1 (n=43) and cohort 2 (n=180), median CrAg titers were 1:10 (IQR 1:5 – 1:20) in the CrAgSQ 1+ category; 1:40 (IQR 1:20 – 1:80) in the CrAgSQ 2+

category; 1:640 (IQR 1:160 – 1:2560) in the CrAgSQ 3+ category; and 1:5120 (IQR 1:2560 – 1:30720) in the CrAgSQ 4+ category (Figure 3).

Among the 189 CrAgSQ-positive patients included in the two CrAg screening studies (excluding the cryptococcal meningitis patients enrolled in the treatment trial) the prevalence of CNS involvement at baseline was strongly associated with CrAgSQ score. Cryptococcal meningitis was confirmed at baseline in 3.8% (3/80) in the CrAgSQ 1+ category, 17.7% (3/17) in the 2+ category, 16.7% (12/72) in the 3+ category, and 80% (16/20) in the 4+ category, p-value for trend <0.001 (Figure 4); it is important to note that these are minimum estimates as LP was only performed in approximately one third of patients (primarily because patients declined the investigation), and possibly performed more frequently in those with symptoms of CNS disease. CrAgSQ score was also strongly associated with mortality. Overall in the combined cohorts 1 and 2, 10 week mortality was 2.0% (11/554) in CrAgSQ-ve individuals; 5.1% (4/78) in those with CrAgSQ 1+ scores; 11.8% (2/17) with CrAgSQ 2+ scores; 18.8% (16/85) with CrAgSQ 3+ scores; and 45.2% (19/42)with CrAg 4+ scores (Table 3), p<0.0001. Restricting analysis to participants in the two CrAg screening studies (excluding the cryptococcal meningitis patients enrolled in the treatment trial), 10 week mortality was 2.0% (11/554) in CrAgSQ-ve individuals; 2.9% (2/68) in those with CrAgSQ 1+ scores; 13.3% (2/15) with CrAgSQ 2+ scores; 16.1% (9/56) with CrAgSQ 3+ scores; and 53.3% (8/15) with CrAg 4+ scores (Table 3), p<0.001. Findings in sensitivity analyses where those lost to follow-up were assumed to have died were unchanged (Table 3).

DISCUSSION

The novel CrAgSQ semi-quantitative CrAg assay had high sensitivity and specificity when compared to the current gold-standard CrAg LFA test. Inter-rater agreement in reading the semi-quantitative results was excellent, and the test provided rapid and reliable estimation of CrAg titers. Increasing CrAgSQ

scores were strongly associated with presence of CNS involvement in CrAg-positive individuals identified through CrAg screening programs, and with acute mortality.

The role of CrAg titers in guiding clinical management of HIV-positive patients with asymptomatic cryptococcal antigenemia identified through CrAg screening programs and in those with overt clinical cryptococcal meningitis has yet to be defined. Accumulating clinical data suggests that quantification of CrAg levels using tests such as the CrAgSQ could enable stratification of patients into differentiated diagnostic and treatment pathways. Recent data from CrAg screening programs in Africa have shown that asymptomatic CrAg-positive individuals with CD4 cell counts below 200 cells/µL have mortality rates two- to three-fold higher than their CrAg-negative counterparts with similar CD4 counts (2, 23, 24), despite treatment with high dose oral fluconazole therapy as recommended in WHO guidelines (16). This is likely to be due in part to the presence of CNS disease, detectable by LP and CSF evaluation, in approximately one-third of asymptomatic CrAg-positive patients with advanced HIV (18) for which fluconazole monotherapy is likely to be insufficient to effectively clear infection (25, 26). However, even CrAg-positive individuals without CNS involvement at baseline have been shown to progress to cryptococcal meningitis and death despite high dose fluconazole therapy (27), suggesting that a proportion of CrAg-positive patients without overt CNS disease may benefit from the intensified antifungal regimens recommended for the treatment of cryptococcal meningitis (16, 28). Conversely, it is well established that a sizeable proportion of asymptomatic CrAg-positive individuals identified through screening programs (approximately 50%) can clear their cryptococcal antigenemia with effective ART induced immune reconstitution alone (13).

Identifying which CrAg-positive individuals require investigation for CNS disease and / or more intensive antifungal therapy regimens, and who can be managed effectively with oral fluconazole alone, is therefore a critical question for CrAg screening programs (16). Elevated CrAg titers of >1:160 have been shown to be highly predictive of prevalent CNS disease at the time of CrAg screening (with a sensitivity

of 88% and specificity of 82%) in a study from South Africa (18), with similar findings reported in Ethiopia (29). Elevated CrAg titers of ≥1:80 or ≥1:160 have also been shown to be strongly associated with increased risk of mortality in CrAg-positive populations (2, 19). Our findings that higher CrAgSQ antigen quantification scores are strongly associated with both CNS involvement at baseline in CrAg-positive individuals, and with higher mortality at ten weeks, add to the already compelling evidence that CrAg titer data could be used to risk stratify CrAg-positive individuals and guide treatment. Although further data are required to inform definitive clinical guidelines, a preliminary suggestion based on our data could be that individuals with a CrAgSQ 1+ score, in whom the risk of baseline CNS involvement and acute mortality is very low could be managed as per current guidelines with high dose fluconazole and ART alone, without the need for LP. Those with CrAgSQ scores of 2+ to 3+ could undergo more intensive clinical evaluation and / or receive more intensive antifungal therapy, for example a combination of fluconazole and flucytosine (4); whilst those with CrAgSQ scores of 4+, who are at extremely high risk of CNS disease and mortality, could be admitted for inpatient evaluation and treatment. Such stratification would enable a large proportion of CrAg-positive individuals to be easily managed as outpatients (43% [80/189] of CrAg-positive individuals from the screening studies included in our analysis had a CrAgSQ 1+ score), and intensive management to be focused on the smaller proportion of individuals at very high risk of complications to reduce the high mortality currently observed in this patient population.

Risk stratification based on CrAg quantification to guide differentiated care in patients presenting with clinical cryptococcal meningitis may also be possible. Extensive data show the strong association between higher baseline CrAg titers in both blood and CSF and subsequent mortality in patients undergoing treatment for HIV-associated cryptococcal meningitis. As new all-oral (4) and short course (4, 21) treatment regimens for cryptococcal meningitis are developed, CrAg quantification could be used to define a patient population who could be discharged from hospital early, or be treated in ambulatory

settings. Those with higher titers may be candidates for future adjuvant treatments (30), or longer courses of therapy.

Our study provides the first evidence for the diagnostic performance of the CrAgSQ in a CrAg screening program targeting individuals with CD4 cell counts ≤200 cells/μL, and also provides information to guide the interpretation of CrAg SQ scores in HIV-infected patients with cryptococcal infection. However, the utility of stratifying patient management based on these scores requires further evaluation in prospective trials. While we have shown associations between CrAgSQ score and the key clinical variables of CNS disease and mortality, our analysis is limited by the relatively low levels of investigation for CNS disease at baseline, and a lack of detailed information regarding adherence to treatment guidelines in the CrAg-positive outpatient population. Our analysis is also unable to provide any information regarding the potential impacts of introducing alternative management strategies according to CrAg based risk stratification, or the cost-effectiveness of these strategies. Although not yet confirmed, preliminary information from IMMY suggest the CrAgSQ test will cost in the range of US$5-6. Finally, the CrAgSQ test identified some patients as positive at 1+ who had negative IMMY CrAg LFA and EIA results. While we have classified these as false positive, further CSF and clinical outcome data on this group are needed in order to determine whether such results represent early cryptococcal infection or not. Many of these false positive samples had EIA optical density readings above zero but below the suggested cut-off, which may represent very low levels of cryptococcal antigenemia. A degree of disparity in test results is inevitable in samples with very low concentrations of the antigen at or near the limit of detection. Notably, none of the patients with positive CrAg SQ and negative IMMY CrAg LFA results developed cryptococcal disease, despite not receiving any antifungal therapy, suggesting that even if the do represent very low CrAg titers, they are of limited clinical significance in individuals who initiate effective antiretroviral therapy.

In conclusion, the semi-quantitative CrAgSQ cryptococcal antigen test had high sensitivity and specificity compared to current IMMY CrAg LFA test and provided quantitative CrAg results which were associated with both CrAg titers derived from dilutional testing and clinical outcomes. The test provides an effective and practical method to stratify CrAg-positive patients according to CrAg levels and could provide the basis for differentiated management approaches to reduce the high mortality seen in HIV-positive patients with cryptococcal infection.

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REFERENCES

1. Rajasingham R, Smith RM, Park BJ, Jarvis JN, Govender NP, Chiller TM, Denning DW, Loyse A, Boulware DR. 2017. Global burden of disease of HIV-associated cryptococcal meningitis: an updated analysis. Lancet Infect Dis 17(8): 873-881.
2. Tenforde MW, Mokomane M, Leeme T, Patel RKK, Lekwape N, Ramodimoosi C, Dube B, Williams EA, Mokobela KO, Tawanana E, Pilatwe T, Hurt WJ, Mitchell H, Banda DL, Stone H, Molefi M, Mokgacha K, Phillips H, Mullan PC, Steenhoff AP, Mashalla Y, Mine M, Jarvis JN. 2017. Advanced Human Immunodeficiency Virus Disease in Botswana Following Successful Antiretroviral Therapy Rollout: Incidence of and Temporal Trends in Cryptococcal Meningitis. Clin Infect Dis 65:779-786.
3. Osler M, Hilderbrand K, Goemaere E, Ford N, Smith M, Meintjes G, Kruger J, Govender NP, Boulle A. 2018. The Continuing Burden of Advanced HIV Disease Over 10 Years of Increasing Antiretroviral Therapy Coverage in South Africa. Clin Infect Dis 66:S118-S125.
4. Molloy SF, Kanyama C, Heyderman RS, Loyse A, Kouanfack C, Chanda D, Mfinanga S, Temfack E, Lakhi S, Lesikari S, Chan AK, Stone N, Kalata N, Karunaharan N, Gaskell K, Peirse M, Ellis J, Chawinga C, Lontsi S, Ndong JG, Bright P, Lupiya D, Chen T, Bradley J, Adams J, van der Horst C, van Oosterhout JJ, Sini V, Mapoure YN, Mwaba P, Bicanic T, Lalloo DG, Wang D, Hosseinipour MC, Lortholary O, Jaffar S, Harrison TS, Team ATS. 2018. Antifungal Combinations for Treatment of Cryptococcal Meningitis in Africa. N Engl J Med 378:1004-1017.
5. Beardsley J, Wolbers M, Kibengo FM, Ggayi AB, Kamali A, Cuc NTK, Binh TQ, Chau NV, Farrar J, Merson L, Phuong L, Thwaites G, Van Kinh N, Thuy PT, Chierakul W, Siriboon S, Thiansukhon E, Onsanit S, Supphamongkholchaikul W, Chan AK, Heyderman RS, Mwinjiwa E, van Oosterhout JJ, Imran D, Basri H, Mayxay M, Dance DA, Phimmasone P, Rattanavong S, Lalloo D, Day JN. 2016.

Adjunctive Dexamethasone in HIV-Associated Cryptococcal Meningitis. N Engl J Med 374:542-54.

6. Patel RKK, Leeme T, Azzo C, Tlhako N, Tsholo K, Tawanana EO, Molefi M, Mosepele M, Lawrence DS, Mokomane M, Tenforde MW, Jarvis JN. 2018. High Mortality in HIV-Associated Cryptococcal Meningitis Patients Treated With Amphotericin B-Based Therapy Under Routine Care Conditions in Africa. Open Forum Infect Dis 5:ofy267.

7. Tenforde MW, Gertz AM, Lawrence DS, Wills NK, Guthrie BL, Farquhar C, Jarvis JN. 2020. Mortality from HIV-associated meningitis in sub-Saharan Africa: a systematic review and meta-analysis. J Int AIDS Soc 23:e25416.

8. Jarvis JN, Harrison TS. 2016. Forgotten but not gone: HIV-associated cryptococcal meningitis. Lancet Infect Dis 16:756-758.

9. Tenforde MW, Jarvis JN. 2019. HIV-associated cryptococcal meningitis: ongoing challenges and new opportunities. Lancet Infect Dis 19:793-794.

10. Jarvis JN, Percival A, Bauman S, Pelfrey J, Meintjes G, Williams GN, Longley N, Harrison TS, Kozel TR. 2011. Evaluation of a novel point-of-care cryptococcal antigen test on serum, plasma, and urine from patients with HIV-associated cryptococcal meningitis. Clin Infect Dis 53:1019-23.

11. Boulware DR, Rolfes MA, Rajasingham R, von Hohenberg M, Qin Z, Taseera K, Schutz C, Kwizera R, Butler EK, Meintjes G, Muzoora C, Bischof JC, Meya DB. 2014. Multisite validation of cryptococcal antigen lateral flow assay and quantification by laser thermal contrast. Emerg Infect Dis 20:45-53.

12. Wake RM, Jarvis JN, Harrison TS, Govender NP. 2018. Brief Report: Point of Care Cryptococcal Antigen Screening: Pipetting Finger-Prick Blood Improves Performance of Immunomycologics Lateral Flow Assay. J Acquir Immune Defic Syndr 78:574-578.

13. Jarvis JN, Lawn SD, Vogt M, Bangani N, Wood R, Harrison TS. 2009. Screening for Cryptococcal Antigenemia in Patients Accessing an Antiretroviral Treatment Program in South Africa. Clin Infect Dis 48:856-862.

14. Jarvis JN, Lawn SD, Wood R, Harrison TS. 2010. Cryptococcal antigen screening for patients initiating antiretroviral therapy: time for action. Clin Infect Dis 51:1463-5.

15. Govender NP, Glencross DK. 2018. National coverage of reflex cryptococcal antigen screening: A milestone achievement in the care of persons with advanced HIV disease. S Afr Med J 108:534-535.

16. WHO. Guidelines for the diagnosis, prevention, and management of cryptococcal disease in HIV-infected adults, adolescents and children, March 2018. Geneva: World Health Organization. Licence: CC BY-NC-SA 3.0 IGO., 2018.

17. Jarvis JN, Bicanic T, Loyse A, Namarika D, Jackson A, Nussbaum JC, Longley N, Muzoora C, Phulusa J, Taseera K, Kanyembe C, Wilson D, Hosseinipour MC, Brouwer AE, Limmathurotsakul D, White N, van der Horst C, Wood R, Meintjes G, Bradley J, Jaffar S, Harrison T. 2013. Determinants of Mortality in a Combined Cohort of 501 Patients with HIV-associated Cryptococcal Meningitis: Implications for Improving Outcomes. Clin Infect Dis.

18. Wake RM, Britz E, Sriruttan C, Rukasha I, Omar T, Spencer DC, Nel JS, Mashamaite S, Adelekan A, Chiller TM, Jarvis JN, Harrison TS, Govender NP. 2018. High Cryptococcal Antigen Titers in Blood Are Predictive of Subclinical Cryptococcal Meningitis Among Human Immunodeficiency Virus-Infected Patients. Clin Infect Dis 66:686-692.

19. Meya DB, Kiragga AN, Nalintya E, Morawski BM, Rajasingham R, Park BJ, Mubiru A, Kaplan JE, Manabe YC, Boulware DR. 2019. Reflexive Laboratory-Based Cryptococcal Antigen Screening and Preemptive Fluconazole Therapy for Cryptococcal Antigenemia in HIV-Infected Individuals With

CD4 <100 Cells/μL: A Stepped-Wedge, Cluster-Randomized Trial. J Acquir Immune Defic Syndr 80:182-189.

20. Lechiile K, Mitchell HK, Mulenga F, Goercke I, Azama MS, Molefi M, Leeme TB, Tenforde MW, Mine M, Jarvis JN. 2017. Prevalence of Advanced HIV Disease and Cryptococcal Infection in Gaborone, Botswana. 24th Conference on Retroviruses and Opportunistic Infections (CROI 2017), February 13-17, 2017 Seattle, WA.

21. Jarvis JN, Leeme TB, Molefi M, Chofle AA, Bidwell G, Tsholo K, Tlhako N, Mawoko N, Patel RKK, Tenforde MW, Muthoga C, Bisson GP, Kidola J, Changalucha J, Lawrence D, Jaffar S, Hope W, Molloy SLF, Harrison TS. 2019. Short-course High-dose Liposomal Amphotericin B for Human Immunodeficiency Virus-associated Cryptococcal Meningitis: A Phase 2 Randomized Controlled Trial. Clin Infect Dis 68:393-401.

22. Cohen J. 1968. Weighted kappa: nominal scale agreement with provision for scaled disagreement or partial credit. Psychol Bull 70:213-20.

23. Longley N, Jarvis JN, Meintjes G, Boulle A, Cross A, Kelly N, Govender NP, Bekker LG, Wood R, Harrison TS. 2016. Cryptococcal Antigen Screening in Patients Initiating ART in South Africa: A Prospective Cohort Study. Clin Infect Dis 62:581-587.

24. Mfinanga S, Chanda D, Kivuyo SL, Guinness L, Bottomley C, Simms V, Chijoka C, Masasi A, Kimaro G, Ngowi B, Kahwa A, Mwaba P, Harrison TS, Egwaga S, Jaffar S, team Rt. 2015. Cryptococcal meningitis screening and community-based early adherence support in people with advanced HIV infection starting antiretroviral therapy in Tanzania and Zambia: an open-label, randomised controlled trial. Lancet 385: 2173-82

25. Longley N, Muzoora C, Taseera K, Mwesigye J, Rwebembera J, Chakera A, Wall E, Andia I, Jaffar S, Harrison TS. 2008. Dose response effect of high-dose fluconazole for HIV-associated cryptococcal meningitis in southwestern Uganda. Clin Infect Dis 47:1556-61.

26. Nussbaum JC, Jackson A, Namarika D, Phulusa J, Kenala J, Kanyemba C, Jarvis JN, Jaffar S, Hosseinipour MC, Kamwendo D, van der Horst CM, Harrison TS. 2009. Combination flucytosine and high-dose fluconazole compared with fluconazole monotherapy for the treatment of cryptococcal meningitis: a randomized trial in Malawi. Clin Infect Dis 50:338-44.

27. Wake RM, Govender NP, Omar T, Nel C, Mazanderani AH, Karat AS, Ismail NA, Tiemessen CT, Jarvis JN, Harrison TS. 2020. Cryptococcal-related mortality despite fluconazole pre-emptive treatment in a cryptococcal antigen (CrAg) screen-and-treat programme. Clin Infect Dis. 70:1683-1690.

28. Tenforde MW, Shapiro AE, Rouse B, Jarvis JN, Li T, Eshun-Wilson I, Ford N. 2018. Treatment for HIV-associated cryptococcal meningitis. Cochrane Database Syst Rev 7:CD005647.

29. Beyene T, Zewde AG, Balcha A, Hirpo B, Yitbarik T, Gebissa T, Rajasingham R, Boulware DR. 2017. Inadequacy of High-Dose Fluconazole Monotherapy Among Cerebrospinal Fluid Cryptococcal Antigen (CrAg)-Positive Human Immunodeficiency Virus-Infected Persons in an Ethiopian CrAg Screening Program. Clin Infect Dis 65:2126-2129.

30. Jarvis JN, Meintjes G, Rebe K, Williams GN, Bicanic T, Williams A, Schutz C, Bekker LG, Wood R, Harrison TS. 2012. Adjunctive interferon-gamma immunotherapy for the treatment of HIV-associated cryptococcal meningitis: a randomized controlled trial. Aids 26:1105-1113.

437 **Tables**

438 **Table 1.** Baseline characteristics of study participants

Variable	**Value** (median, IQR or %, n)
Cohort 1. Sequential cohort of individuals with CD4 cell counts ≤200 cells/µL screened for CrAg (n=638)*	
Age (years)	40 years (IQR 33-46)
Sex (% male)	57% (n=365)
CD4 count (cells/µL)	91 cells/µL (IQR 53-150)
Cryptococcal antigenemia† (% positive)	5.8% (n=37)
Testing location (% outpatients)	88% (n=563)
ART status§ (% on ART)	71% (n=451)
Prior cryptococcal meningitis (%)	2% (n=13)
Cohort 2. Titer validation cohort (all CrAg-positive, n=180)**	
Age (years)	39 years (IQR 34-44)
Sex (% male)	59% (n=104)
CD4 count (cells/µL)	41 cells/µL (IQR 16-85)
Cryptococcal antigenemia† (% positive)	100% (n=180)
Testing location (% outpatients)	49% (n=88)
ART status§§ (% on ART)	46% (n=82)
Prior cryptococcal meningitis (%)	12% (n=21)

IQR: inter-quartile range; n: number; CrAg: cryptococcal antigen; ART: antiretroviral therapy
*Sequential samples from individuals with CD4 cell counts ≤200 cells/µL tested during a reflex CrAg screening program.
†Cryptococcal antigen positive using the IMMY [IMMY, Norman, OK, USA] lateral flow assay.
§ 451/638 (71%) on ART, 32/638 (5%) defaulted ART, 155/638 (24%) ART naïve. Viral loads were available for 448 of those on ART, or whom 168 (38%) had a detectable viral load.
§§ 82/180 (46%) on ART, 13/180 (7%) defaulted ART, 85/180 (47%) ART naïve. Viral loads were

available for 55 of those on ART, of whom 16 (29%) had a detectable viral load.
**The CrAg validation cohort (cohort 2) consisted of 111 known CrAg-positive plasma samples from reflex cryptococcal antigen screening studies and 69 plasma samples from patients with cryptococcal meningitis enrolled in a clinical trial (n=180 total).

439

Table 2. Diagnostic performance of the IMMY semi-quantitative cryptococcal lateral flow assay

A. Inter-rater agreement

		Rater B					
		0	**1+**	**2+**	**3+**	**4+**	Total
Rater A	**0**	610	0	0	0	0	610
	1+	4	87	0	0	0	91
	2+	0	3	15	0	0	18
	3+	0	0	4	101	5	110
	4+	0	0	0	0	43	43
	Total	614	90	19	101	48	872

B. Inter-rater reliability (Cohen's Kappa Statistic)

Expected agreement	Observed agreement	Kappa	Standard error	p-value
52.11%	98.17%	0.962	0.022	<0.0001

C. Weighted* inter-rater reliability (Cohen's Kappa Statistic)

Expected agreement	Observed agreement	Kappa	Standard error	p-value
72.07%	99.54%	0.983	0.028	<0.0001

*To account for the ordered categorical data and assess the *degree* of disagreement, disagreements were weighted in a linear way; with five categories, cases in adjacent categories were weighted by factor 0.75, those with a distance of two categories weighted 0.5, those with a distance of three categories weighted 0.25, and those with a distance of four categories weighted 0.

D. Sensitivity, specificity, positive, and negative predictive value of the IMMY semi-quantitative LFA (CrAgSQ) versus the conventional IMMY LFA test†

		IMMY LFA +ve	**IMMY LFA -ve**	Total
	CrAgSQ +ve	40	40	80
	CrAgSQ -ve	3	609	612
	Total	43	649	692

Sensitivity	93.0%	95% CI 80.9% - 98.5%
Specificity	93.8%	95% CI 91.7% - 95.6%
Positive predictive value	50.0%	95% CI 38.6% - 61.4%

Negative predictive value	99.5%	95% CI 98.6% - 99.9%

E. Sensitivity, specificity, positive, and negative predictive value of the IMMY semi-quantitative LFA (CrAgSQ) versus the conventional IMMY LFA test after reconciliation of discordant tests[§] using EIA testing

	CrAg +ve	**CrAg -ve**	Total
CrAgSQ +ve	53	27	80
CrAgSQ -ve	1	611	612
Total	54	638	692

Sensitivity	98.1%	95% CI 90.1% - 100%
Specificity	95.8%	95% CI 93.9% - 97.2%
Positive predictive value	66.3%	95% CI 54.8% - 76.4%
Negative predictive value	99.8%	95% CI 99.1% - 100%

IMMY: IMMY, Norman, OK, USA ; LFA: lateral flow assay; CrAgSQ: semi-quantitative LFA; EIA: [ADD]

†All CrAgSQ results of 1+ and above were considered positive. The conventional IMMY qualitative lateral flow assay was considered the reference test.

§ All CrAgSQ results of 1+ and above were considered positive. The reference standard was a composite cryptococcal antigen result derived from the conventional IMMY qualitative lateral flow assay with discrepant LFA/SQ results reconciled using the IMMY EIA as the tie-breaker test. See Figure 1 for details.

449 **Table 3.** Associations between IMMY semi-quantitative cryptococcal lateral flow assay titers and
450 mortality

A. All participants (cohorts 1 and 2)				
CrAgSQ Titer	**Mortality***	**Hazard Ratio†**	**95% Confidence Interval**	**p-value**
0	2.0% (11/554)	Base	--	
1+	5.1% (4/78)	2.63	0.84 - 8.26	
2+	11.8% (2/17)	6.26	1.39 – 28.25	<0.0001
3+	18.8% (16/85)	10.18	4.73 – 21.95	
4+	45.2% (19/42)	28.85	13.70 – 60.75	
CrAgSQ Titer	**Dead or lost to follow-up§**	**Hazard Ratio†**	**95% Confidence Interval**	**p-value**
0	4.9% (28/571)	Base	--	
1+	6.3% (5/79)	1.29	0.50 – 3.34	
2+	11.8% (2/17)	2.45	0.59 – 10.31	<0.0001
3+	19.8% (17/86)	4.12	2.30 – 7.67	
4+	52.1% (25/48)	14.1	8.19 – 24.17	
B. Participants in CrAg screening studies				
CrAgSQ Titer	**Mortality***	**Hazard Ratio†**	**95% Confidence Interval**	
0	2.0% (11/554)	Base	--	
1+	2.9% (2/68)	1.75	0.50 – 6.13	
2+	13.3% (2/15)	5.70	1.29 – 25.27	<0.0001
3+	16.1% (9/56)	6.85	3.00 – 15.62	
4+	53.3.% (8/15)	31.80	13.01 – 77.31	

CrAgSQ Titer	Dead or lost to follow-up[§]	Hazard Ratio[†]	95% Confidence Interval
0	4.9% (28/571)	Base	--
1+	4.4% (3/69)	0.97	0.35 – 2.77
2+	13.3% (2/15)	2.38	0.57 – 9.94
3+	17.5% (10/57)	3.11	1.57 – 6.20
4+	65.0% (13/20)	18.33	9.54 – 35.22

IMMY: IMMY, Norman, OK, USA; CrAgSQ: semi-quantitative lateral flow assay
*Mortality at 10 weeks. Loss to follow-ups censored.
†Derived from Cox proportional hazards model.
[§]Dead or lost to follow-up at 10 weeks. Twenty-five patients (3%) were lost to follow-up prior to 10 weeks.

458 **Figure Legend**

459 **Figure 1.** The IMMY semi-quantitative CrAgSQ lateral flow assay (IMMY, Norman, OK, USA). Samples are
460 diluted 1:1 with specimen diluent prior to testing (as is also the case with the conventional lateral flow
461 assay). Scores indicating increasing cryptococcal antigen titers are derived from line intensity patterns as
462 follows: T1<T2 = 1+; T1=T2 = 2+; T1>T2 = 3+; only T1 = 4+; only C = 5+. Only T2 and C = negative. A score
463 of 1+ indicates “low positive”, and a score of 5+ “very high positive”.

464

465 **Figure 2.** Schema of plasma samples and patient populations used in the diagnostic validation study.

466

467 **Figure 3.** Relationship between CrAgSQ scores and cryptococcal antigen titers derived from serial
468 dilutional testing with the IMMY lateral flow assay (Panel 1). Samples with discordant CrAgSQ and IMMY
469 lateral flow assay positive / negative results were retested using the IMMY cryptococcal antigen enzyme
470 immunoassay (EIA) (panel 2). Box A indicates samples that were positive on IMMY lateral flow assay
471 testing and negative on CrAgSQ testing. Three of these four samples were negative on EIA testing at the
472 optical density cut off of 0.265. Box B indicates the samples that were positive on CrAgSQ testing and
473 negative on IMMY lateral flow assay testing. Thirteen of the forty samples were positive on EIA testing
474 at the optical density cut off of 0.265.

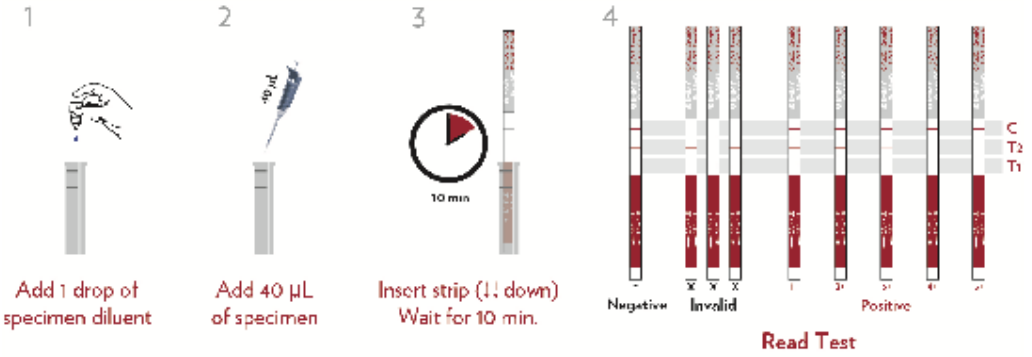
475

476 **Figure 4.** Associations between CrAgSQ score and A) baseline CNS disease (defined as positive
477 cerebrospinal fluid CrAg) in the 189 CrAg-positive patients identified through reflex cryptococcal antigen
478 screening; B) ten-week mortality in all participants; and C) ten-week mortality and loss to follow-up.
479 Note that only 32% of CrAg-positive individuals underwent baseline CSF examination, thus these figures
480 represent *minimum* estimates of baseline CNS disease.

481

482 **Figures**

483 **Figure 1**



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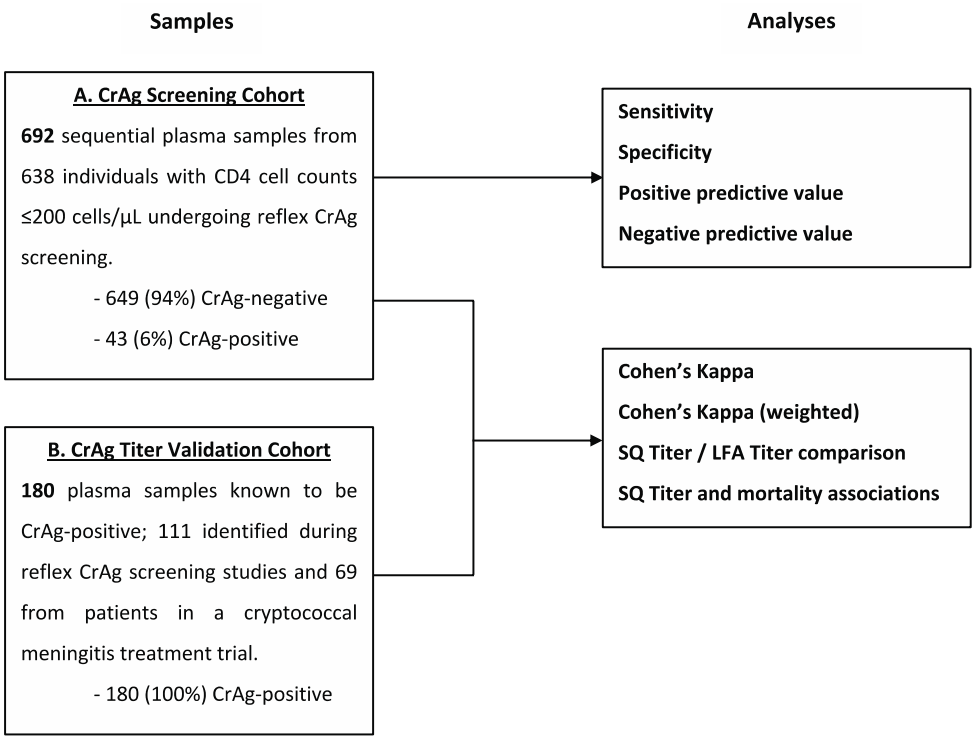
487 **Figure 2**



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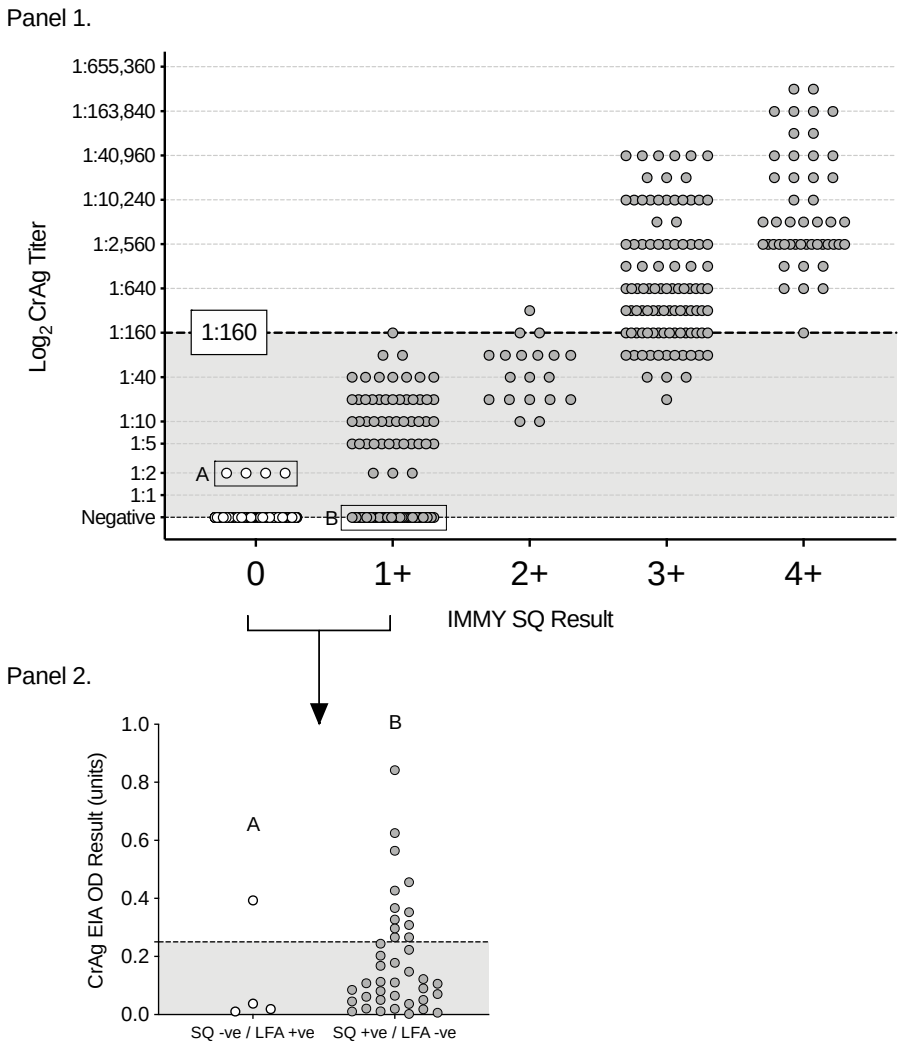
489 **Figure 3**



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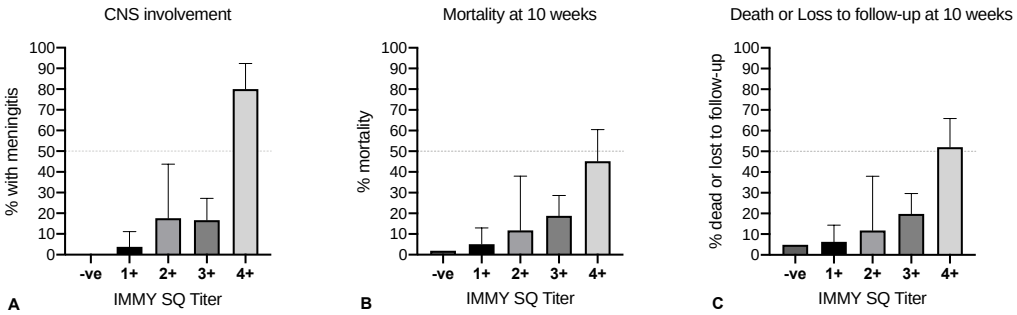
492 **Figure 4**



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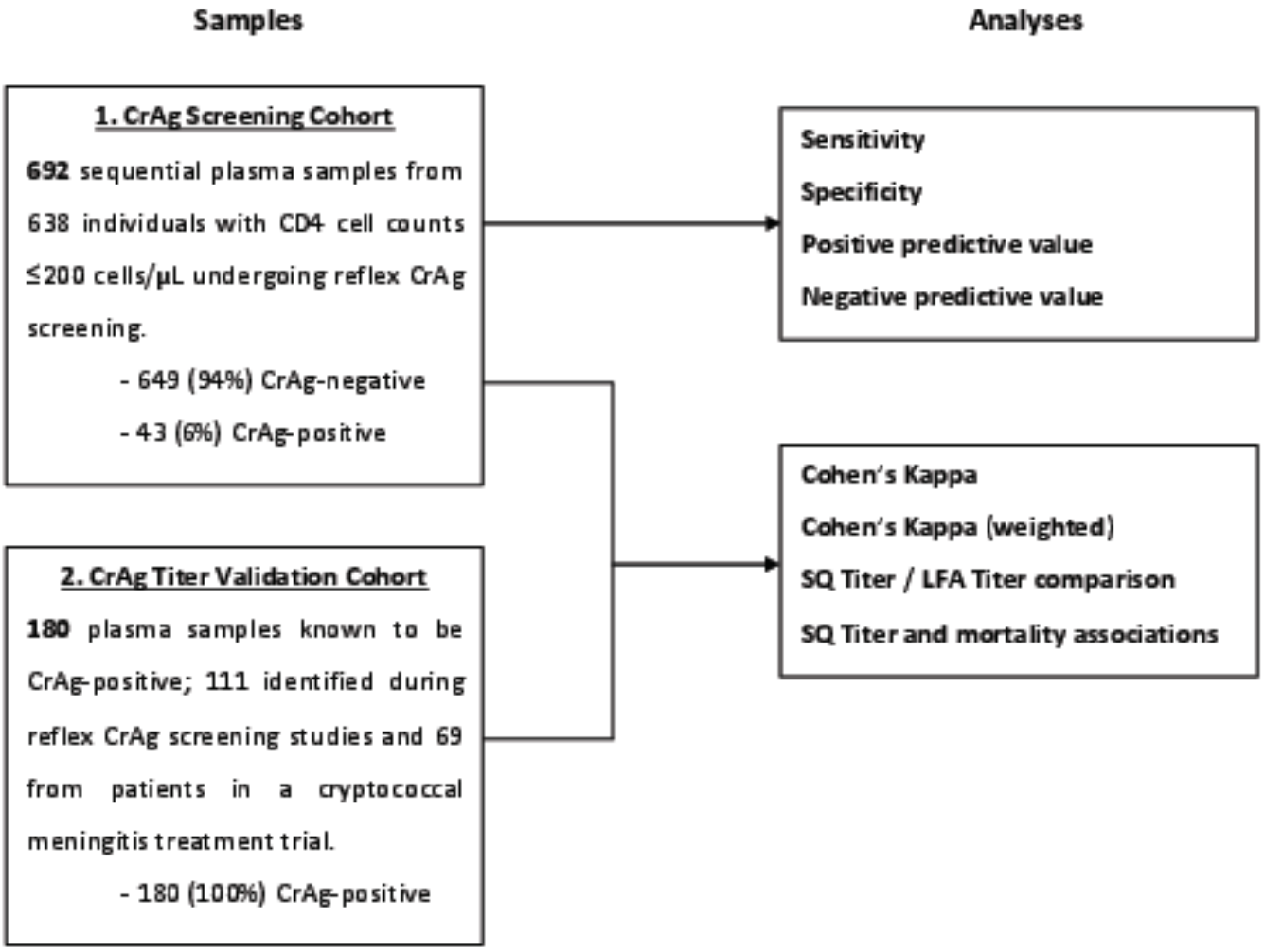

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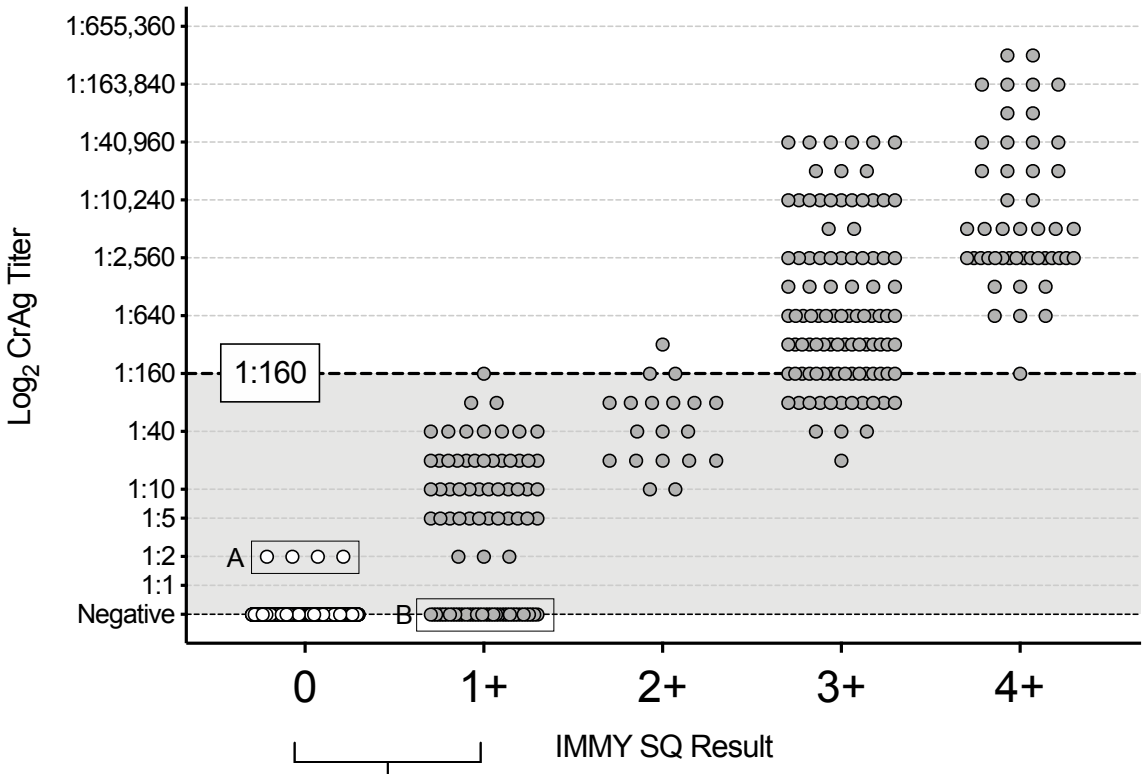

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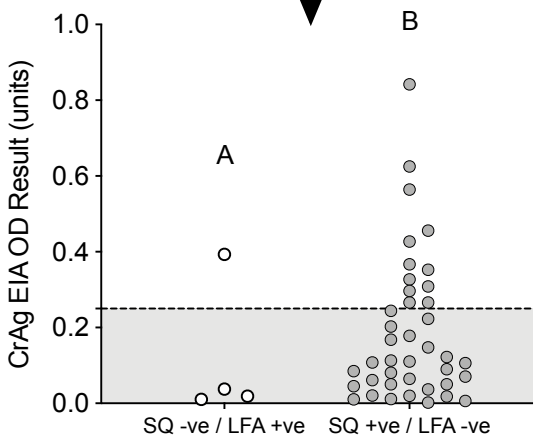

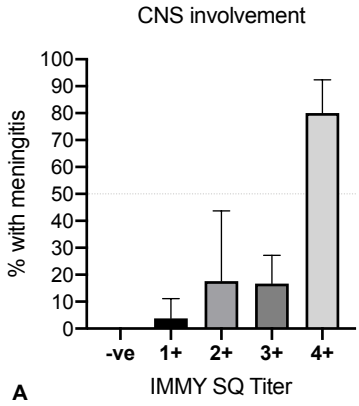

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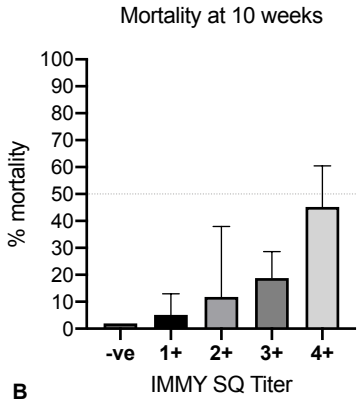

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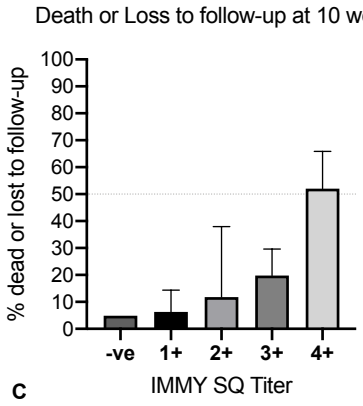

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