The association of antibody immunity with cryptococcal antigenemia and mortality in a South African cohort with advanced HIV disease

Hyunah Yoon[1]*, Rachel M. Wake[2,3,4]*, Antonio S. Nakouzi[1,5], Tao Wang[6], Ilir Agalliu[6,7], Caroline T. Tiemessen[8,9], Nelesh P. Govender [2,3,8,10,11], Joseph N. Jarvis[12,13], Thomas S. Harrison[2,4,10], Liise-anne Pirofski[1,5]

1. Division of Infectious Diseases, Department of Medicine, Albert Einstein College of Medicine, Montefiore Medical Center, Bronx, New York, USA
2. Institute for Infection and Immunity, St George's University Hospital NHS Foundation Trust, London, UK
3. Centre for Healthcare-Associated Infections, Antimicrobial Resistance and Mycoses, National Institute for Communicable Diseases, a Division of the National Health Laboratory Service, Johannesburg, South Africa
4. Clinical Academic Group in Infection and Immunity, St George's University Hospital NHS Foundation Trust, London, UK
5. Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York, USA
6. Department of Epidemiology and Population Health, Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, New York, USA
7. Department of Urology, Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, New York, USA
8. Faculty of Health Sciences, University of the Witwatersrand, South Africa

9. Centre for HIV & STIs, National Institute for Communicable Diseases, a Division of the NHLS, South Africa
10. MRC center for Medical Mycology, University of Exeter, Exeter, UK
11. Division of Medical Microbiology, University of Cape Town, South Africa
12. Department of Clinical Research, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, United Kingdom
13. Botswana Harvard AIDS Institute Partnership, Botswana

***HY and RW contributed equally to this manuscript.**

Corresponding author:

Liise-anne Pirofski, MD

Division of Infectious Diseases, Department of Medicine

Albert Einstein College of Medicine

1300 Morris Park Ave, Room 610

Belfer Building, Bronx, NY, USA 10461

l.pirofski@einsteinmed.edu

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Abstract

Background:

Asymptomatic cryptococcal antigenemia (positive blood cryptococcal antigen [CrAg]) is associated with increased mortality in HIV-positive individuals even after adjusting for CD4 count and despite receiving antifungal treatment. The association of antibody immunity with mortality in HIV-positive adults with cryptococcal antigenemia is unknown.

Methods:

Cryptococcus spp. capsular glucuronoxylomannan (GXM)- and naturally occurring β-glucans (laminarin, curdlan)-binding antibodies were measured in blood samples of 197 South Africans living with HIV who underwent CrAg screening and were prospectively followed up to 6 months. Associations between antibody titers, CrAg status and all-cause mortality were sought using logistic and Cox proportional hazards regression, respectively.

Results:

Compared to CrAg-negative (n=130), CrAg-positive individuals (n=67) had significantly higher IgG1 (median, 6,672 [interquartile range, IQR, 4,696-10,414] vs. 5,343 [3,808-7,722]μg/ml; *P*=0.007), IgG2 (1,467 [813-2,607] vs. 1,036 [519-2,012]μg/ml; *P*=0.01), GXM-IgG (1:170 [61-412] vs. 1:117 [47-176]; *P*=0.0009) and lower curdlan-IgG (1:47 [11-133] vs. 1:93 [40-206]; *P*=0.01) titers. GXM-IgG associated directly with cryptococcal antigenemia adjusted for CD4 count and antiretroviral therapy use (odds ratio, 1.64 for each log increase in titer; 95% confidence interval [CI], 1.21-2.22). Among CrAg-positive individuals, GXM-IgG inversely associated with mortality at 6 months adjusted for CD4 count and active or prevalent tuberculosis (hazard ratio, 0.50; a two-fold reduction per log increase in titer; 95% CI, 0.33-0.77).

Conclusions:

The finding of an inverse association of GXM-IgG with mortality in CrAg-positive individuals suggests that GXM-IgG titer may have prognostic value in such individuals. Prospective longitudinal studies of antibody levels to investigate this hypothesis and identify mechanisms by which antibody may protect against mortality are warranted.

Keywords: cryptococcal antigenemia, HIV/AIDS, serology, GXM-IgG, mortality

Introduction

Despite an ongoing international effort to end cryptococcal-associated deaths [1], cryptococcosis remains a major cause of morbidity and mortality among people living with human immunodeficiency virus (HIV). Most cases occur in sub-Saharan Africa, where substantial research efforts have advanced treatment and prevention of cryptococcosis [2, 3]. The implementation of cryptococcal antigen (CrAg) screening in individuals with CD4 counts <200 cells/mm^3 makes it possible to identify HIV-positive individuals at risk for developing cryptococcal meningitis (CM) before the onset of symptoms, thereby facilitating earlier diagnostic testing and treatment [2].

While the incidence of CM has declined with CrAg screening and preemptive treatment programs, mortality remains higher in HIV-positive individuals with asymptomatic cryptococcal antigenemia than those without cryptococcal antigenemia, even after adjustment for CD4 count and despite preemptive antifungal therapy [3, 4]. Non-CD4 T-cell associated factors are likely to affect the pathogenesis of cryptococcosis since cryptococcosis occurs in HIV-positive individuals with CD4 T-cell counts >200 cells/mm^3 [5] and in HIV-negative individuals [6]. Previous studies show that *Cryptococcus neoformans* (CN) capsular glucuronoxylomannan (GXM) and β-glucan (laminarin, a branched β-[1-3]-glucan and curdlan, a linear β-[1-3]-glucan)-binding antibody levels associate with CrAg or CM status in HIV-infected and HIV-uninfected individuals [6-9]. In this study, we investigated associations between antibody immunity, cryptococcal antigenemia, and mortality in a South African cohort with advanced HIV disease with and without asymptomatic cryptococcal antigenemia.

Methods

Study design. This is a retrospective study of serum samples obtained at enrollment of a prospective cohort study of asymptomatic CrAg-positive and CrAg-negative HIV-positive adults followed over 6 months after CrAg screening at two large urban hospitals in Johannesburg, South Africa [4]. Participants were screened for asymptomatic/subclinical CM (positive cerebrospinal fluid [CSF] using culture, India Ink microscopy, or CrAg test) when logistically possible. All participants were started on antifungal therapy, and then antiretroviral therapy (ART) as per local management guidelines (National Department of Health, South Africa, 2015). Baseline demographic, clinical, and mortality data for up to 6 months were collected.

Study population. Eligible individuals were HIV-positive adults ≥18 years with CD4 count <100 cells/mm^3. Consecutive CrAg-positive and 1:2 ratio of CrAg-negative individuals attending study sites following CrAg screening were invited to participate. Exclusion criteria were a previous diagnosis of CM, or symptoms or signs of cryptococcal meningitis.

Sample collection and processing. Serum samples collected at baseline were frozen and stored at -20 ^{o}C. Before analysis, they were heat-inactivated at 56 ^{o}C for 30 minutes to inactivate HIV and/or other heat-sensitive viruses as well as complement and stored at 4 ^{o}C.

Antibody measurements. Serum immunoglobulin (Ig), cryptococcal capsular GXM polysaccharide-, and β-glucan (laminarin, curdlan)-binding antibodies were analyzed. The latter bind conserved microbial determinants that are found on the cryptococcal cell wall and that of other fungi [10]. IgM, IgG1, and IgG2 concentrations were measured using a Luminex platform

(Austin, TX) and quantified in units of μg/ml as previously described [7, 11]. GXM-IgM/IgG/IgA and β-glucan-binding IgM/IgG were detected by antigen-capture enzyme-linked immunosorbent assays (ELISA) using plates coated, respectively, with GXM (from CN strain 24067) and laminarin or curdlan and reported as inverse titers as previously described [7, 11]. These assays have been used extensively for the detection of human GXM- and β-glucan-binding antibodies [11-15].

Ethics. Samples were obtained during a cohort study [4], approved by the Ethics Committees at the University of the Witwatersrand and the London School of Hygiene and Tropical Medicine. All patients provided written or witnessed verbal informed consent, and de-identified serum samples were studied under an Albert Einstein College of Medicine Institutional Review Board-approved protocol (1989-228). Samples were transported outside of South Africa with the permission of the local research ethics committee.

Statistical analysis.

Bivariate analysis. Baseline demographic, clinical characteristics, and antibody levels stratified by plasma CrAg status were compared using the Wilcoxon rank-sum test for continuous and Fisher's exact or χ^2 test for categorical variables. All tests were 2-sided (α=0.05). Correlations between measured antibody variables and between antibody variables and plasma CrAg titer were assessed using Spearman's correlation coefficient.

Model building. Association between antibody levels and cryptococcal antigenemia was estimated using multivariable logistic regression and association between antibody levels and

time to mortality was estimated using Cox proportional hazards regression. *A priori* decision was made to include CD4 count in the model. Other variables were included in the full model if *P*-value <0.20 in the univariable model. Variables assessed include ART use prior to enrollment, tuberculosis diagnosed within 6 months prior to, or on the day of enrollment, and log-transformed antibody levels. Total IgG1 or IgG2 were not included in the full model as they may biologically be in the same causal pathway as the antigen-specific antibodies. Variables were selected by backward elimination, checked for confounding and interaction with the main independent variable of interest, GXM-IgG. In the survival analysis, GXM-IgG was evaluated as both a continuous and a categorical variable in tertiles to demonstrate dose-response as Kaplan-Meier curves. Hosmer-Lemeshow goodness of fit and Schoenfeld residuals were assessed in the logistic regression and Cox model, respectively.

Mediation analysis. We fitted a linear regression model for the mediator (log of GXM-IgG) with the exposure status (CrAg positive vs. negative) as the independent variable. The Cox proportional hazards model for the outcome (mortality by 6 months) was built with the exposure status and the mediator as independent variables and potential confounders as covariables. The model also included the exposure-mediator interaction term to assess for potential interactions. Regmedint package in R was used for the analysis [16, 17].

Principal component analysis (PCA). Baseline antibody markers were analyzed using PCA to reduce complex correlated datasets into a series of linear, non-correlated principal components (PCs) and avoid multiple comparisons as described [6]. Log-transformed antibody markers were

normalized to the mean. Differences in top PC scores by CrAg status (positive vs. negative) and mortality were examined using a linear regression model.

All statistical analyses were performed in R and Stata/IC 16.1 software (College Station, TX: StataCorp LLC).

Results:

Study cohort. Demographics and clinical characteristics of the 130 CrAg-negative and 67 CrAg-positive cases are shown in Table 1. The median age was 39 years, 51% were male, 97% self-identified as Black race, with no difference between the CrAg status groups. Compared to CrAg-negative individuals, CrAg-positive individuals had lower CD4 counts (median 27 vs. 40.5 cells/mm^3; *P*=0.003) and a greater proportion were on ART prior to enrollment (23.9% vs. 10.0%; *P*=0.01). The median plasma CrAg titer was 1:40 (interquartile range [IQR], 5-320). The prevalence of tuberculosis within the past 6 months did not differ between the groups. There were significantly more deaths in the CrAg-positive than CrAg-negative group (25.4% vs. 8.5%; hazard ratio [HR], 3.43; 95% confidence interval [CI], 1.61-7.33) (Table 1, Supplementary Table 1).

Antibody marker levels. In the bivariate analysis, CrAg-positive individuals had higher inverse titers of GXM-IgG (median, 169.5 [IQR, 61.1-411.9] vs. 117.3 [47.0-176.3]; *P*=0.0009) and concentrations of IgG1 (6,672.2 [4,695.5-10,414] vs. 5,343.4 [3,807.7-7,721.9] μg/ml; *P*=0.007) and IgG2 (1,467.4 [812.8-2,607] vs. 1,036.0 [519.2-2,012.4] μg/ml; *P*=0.01), and lower curdlan-IgG (46.6 [11.1-133.0] vs. 93.2 [39.6-205.9]; *P*=0.01) than CrAg-negative individuals (Figure 1).

There were no between-group differences in laminarin-binding antibodies. There was collinearity among GXM-IgG, GXM-IgM, and GXM-IgA (Supplementary Figure 1).

Associations of antibody markers with CrAg status. Higher GXM-IgG and lower curdlan-IgG were each associated with CrAg-positive status adjusted for CD4 count and ART use prior to enrollment in the multivariable logistic regression model (adjusted odds ratio [aOR], 1.64; 64% increase in odds per log increase in GXM-IgG titer; 95% CI, 1.21-2.22 and aOR, 0.71; 29% reduction in odds per log increase in curdlan-IgG titer; 95% CI, 0.58-0.87, respectively) (Figure 2).

Correlation between plasma CrAg titer and antibody levels. In the CrAg-positive group (n=67), there were inverse correlations between CrAg titer and IgG1 (ρ=-0.33; *P*=0.007), IgG2 (ρ=-0.29; *P*=0.02), laminarin-IgG (ρ=-0.24; *P*=0.05), and while not significant, GXM-IgG (ρ=-0.19; *P*=0.13) (Supplementary Table 2).

Associations of antibody markers with mortality. Variables significantly associated with death within 6 months in the univariable analysis included: positive plasma CrAg (HR, 3.43; 95% CI, 1.61-7.33), CrAg titer (HR, 1.19; 19% increase in hazard per log increase in titer; 95% CI, 1.05-1.36), tuberculosis diagnosis within the prior 6 months (HR, 2.66; 95% CI, 1.26-5.59), and GXM-IgM (HR, 0.67; 33% decrease in hazard per log increase in titer; 95% CI, 0.45-1.00) (Supplementary Table 1). In the multivariable Cox proportional hazards analysis, there was an interaction between CrAg status and GXM-IgG (interaction term, *P*=0.04), so the model was stratified by CrAg status. In CrAg-positive individuals, GXM-IgG was inversely associated with

mortality in both unadjusted (HR, 0.55; a 1.8-fold reduction per log increase in titer; 95% CI, 0.36-0.84) and adjusted (HR, 0.50; a two-fold reduction per log increase in titer; 95% CI, 0.33-0.77) model for CD4 count and tuberculosis (Table 2, Figure 3). GXM-IgG was not associated with mortality in the CrAg-negative cohort. GXM-IgM was not associated with mortality in the multivariable model.

Mediation analysis. Mediation analysis [16, 18] was performed to examine whether the influence of the exposure (CrAg status) on the potential mediator (GXM-IgG) explains some or all of the significance of the association between an exposure and an outcome (mortality) statistically. A significant interaction between the exposure and mediator variable was detected and CrAg status was significantly associated with GXM-IgG and mortality. The direct effect of CrAg status on mortality was 1.71 (95% CI, 0.88, 2.54), the indirect effect mediated by GXM-IgG was -0.35 (95% CI, -0.67, -0.03), and -57% of the total effect on mortality was mediated by GXM-IgG (95% CI, -1.21, 0.08) (Supplementary Figure 2).

Principal component analysis. Most of the variance in the dataset was explained by PC1 (35%) and PC2 (19%), which were used to explore associations between antibody markers and CrAg status and mortality. PC1 was composed primarily of GXM-IgA, GXM-IgG, and GXM-IgM. PC2 was composed of curdlan-IgG, laminarin-IgG and a negative loading of laminarin-IgM (Supplementary Figure 3). Among CrAg-positive individuals, PC1 score had a significant inverse association with 6-month mortality; mean PC1 score was 0.32 among the CrAg-positive individuals who survived, and -0.66 among the CrAg-positive individuals who died, a difference of 0.98; 95% CI, 0.04, 1.92; *P*=0.04, indicating that higher levels of GXM-IgA, GXM-IgG, and

GXM-IgM were associated with higher survival rates in this population (Supplementary Table 3, Supplementary Figure 4). There was no association between PC score and mortality in CrAg-negative group. No association was found between PC score and CrAg status in the overall cohort.

Discussion

In this retrospective serological study of HIV-positive CrAg-positive and negative participants with CD4 <100 cells/mm3, higher enrollment GXM-IgG and lower curdlan-IgG titers were associated with CrAg-positive status, but not CrAg titer. In addition, for CrAg-positive participants, enrollment GXM-IgG, prior to antifungal therapy, was inversely associated with mortality and a partial mediator on the causal pathway between CrAg and mortality by 6 months. This suggests that lower levels of GXM-IgG may in part contribute to excess mortality in CrAg-positive HIV-positive individuals [3, 4] and echoes a pre-AIDS-era study in which an absence of cryptococcal antibodies was a poor prognostic factor in patients with cryptococcal meningitis [19].

Our data show that HIV-positive CrAg-positive participants with CD4 counts <100 cells/mm^3 had higher IgG1 and IgG2 levels than CrAg-negative participants. However, IgG2 levels in both CrAg-positive and CrAg-negative participants were lower than normal HIV-negative adult values. HIV is associated with hypergammaglobulinemia, generally manifested as increased IgG1, but IgG2 is decreased [20]. IgG2 is the predominant IgG subclass of human antibodies to capsular polysaccharides, including GXM [13, 21]. Thus, as in a prior study [7], higher IgG2 levels in CrAg-positive than CrAg-negative participants may reflect a cryptococcal response. We did not determine GXM-IgG subclasses in this study.

Higher GXM-IgG in CrAg-positive participants parallels multiple previous studies that found higher GXM-IgG titers in HIV-positive and HIV-negative individuals with, than without, cryptococcosis [7-9, 15]. Notably, in participants with cryptococcal antigenemia, lower GXM-IgG was associated with mortality, while in an unadjusted analysis of the entire cohort, lower GXM-IgM was associated with mortality. These associations were supported by the PCA, in which the top principal component, primarily GXM-binding antibodies, was inversely associated with CrAg titer and mortality in CrAg-positive individuals, although the GXM-IgG specific signal in predicting CrAg-positive status was reduced by adding noise from GXM-IgM and GXM-IgA. While association is not causation, these data suggest that GXM-binding antibodies may have a protective role in those with cryptococcal antigenemia. This is consistent with a large body of data showing that GXM monoclonal antibodies (mAbs) mediate beneficial effects, including anticryptococcal activity *in vitro* [22], GXM clearance, including in a clinical trial in patients with cryptococcal meningitis [23], and protection against lethal cryptococcal infection in experimental models [24].

As previously reported [7], we found CrAg-negative participants had detectable GXM-binding antibodies. Most likely, this reflects cryptococcal infection early in life or latency [25] and/or ongoing environmental exposure, though we cannot rule out cross-reactivity with an unknown determinant [21]. GXM-binding antibodies have been detected in HIV-negative and HIV-positive individuals with and without a history of cryptococcosis as well as young children [8, 14]. Thus, higher GXM-IgG titers in CrAg-positive compared to CrAg-negative individuals most likely reflect an immune response to fungal growth. HIV infection impairs responses to new, but not previously encountered antigens [20]. Although fungal growth is not always detected (or quantified if present) in CrAg-positive persons, it is logical to posit that serum

cryptococcal antigenemia emerges when host defense is unable to contain latent or newly acquired cryptococcal infection. Thus, an increase in GXM-IgG might signal cryptococcosis risk in CrAg-negative individuals. Consistent with this idea, anti-mannan antibodies, which can protect mice against experimental candidiasis [26], were detected in patients with candidiasis prior to the development of positive blood cultures [27].

β-glucan-binding antibodies are part of the natural serum antibody repertoire, which binds conserved microbial determinants found on the cryptococcal cell wall and other fungi and is the first line of defense against pathogens [10, 28]. Although we did not find associations between laminarin- or curdlan-binding antibodies and mortality, lower curdlan-IgG was associated with CrAg status and laminarin-IgG inversely correlated with CrAg titer, a correlate of risk for meningitis [29] and death [30]. Previous reports show associations between lower laminarin-antibody titers and CrAg-positive status [7] as well as HIV-associated cryptococcus-associated immune reconstitution inflammatory syndrome [11]. Evidence that naturally occurring β-glucan-binding antibodies could contribute to host defense against cryptococcosis includes that laminarin-binding antibodies have direct anti-cryptococcal effects *in vitro* [31], indirect effects on the inflammatory response [11], and protect mice *in vivo* [32, 33]. Similar to our findings in CrAg-positive participants, candidemic patients had higher β-glucan-associated-protein MP65 titers than non-candidemic patients and survivors had higher titers than non-survivors [34].

We found increased mortality in CrAg-positive participants with tuberculosis and lower curdlan-IgM in participants with than without tuberculosis (Supplementary Table 4). Notably, mycobacterial cell walls contain multiple glucans [35] and decreased levels of serum mycobacterial cell wall-binding IgG were associated with disseminated tuberculosis [36]. Given

that tuberculosis is an independent risk factor for cryptococcosis [37, 38], more work is needed to identify possible shared susceptibility factors.

Strength and limitations

We used well-established antigen-capture ELISAs to detect GXM-, laminarin-, and curdlan-binding antibodies [9, 12, 15]. While previous studies examined the relationship between antibody markers and cryptococcal disease status, we sought associations between antibody markers and all-cause mortality. We used mediation analysis to establish that GXM-IgG partially mediated a causal association between CrAg and mortality and PCA to eliminate multicollinearity between antibody variables. Use of these platforms led to a novel result that implicates GXM-binding antibody levels in survival of HIV-positive CrAg-positive individuals.

Our study also has limitations. Serum CrAg may have affected GXM-binding antibody measurements, we did not assay for immune complexes. Since our assays were performed on a single sample from one point in time, our data does not address longitudinal or temporal variations in antibody levels. We did not perform functional assays and did not investigate the relationship between B cells and cryptococcal disease status [39] or antibody levels as we did not have access to peripheral blood mononuclear cells. Misclassification bias is possible. Though rare, cryptococcal meningitis can occur in CrAg-negative individuals [40] and this may have affected the difference in antibody levels by CrAg status. ART use prior to enrollment associated with cryptococcal antigenemia, most likely reflecting prolonged immune suppression in ART-experienced, non-adherent individuals. While we adjusted for ART use, our understanding of possible interactions between ART and antibody levels is limited. There may have been other

unmeasured or latent confounders that could shed more light on the association between antibody markers, ART, and mortality.

Conclusion:

Levels of IgG1, IgG2, GXM-IgG and β-glucan binding antibodies differed in CrAg-positive and CrAg-negative individuals, GXM-IgG associated with CrAg status as did lower curdlan-IgG, and lower GXM-IgG associated with an increased risk of all-cause mortality in CrAg-positive individuals. These findings support the hypothesis that GXM- and β-glucan-binding antibodies may enhance cryptococcal host defense, highlighting the need for mechanistic and larger-scale prospective longitudinal clinical studies. Such studies may provide a better understanding of the antifungal activities of GXM- and β-glucan-binding antibodies, identify much needed biomarkers of cryptococcal pathogenesis and mortality, and inform opportunities for earlier risk stratification and therapeutic intervention.

NOTES

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Author contributions.

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References

1. Ending Cryptococcal Meningitis Deaths by 2030 – Strategic Framework. South Africa; 2021. https://dndi.org/wp-content/uploads/2021/05/EndCryptococcalMeningitisDeaths2030-StrategicFramework-EN-2021.pdf. Accessed July 6, 2022.
2. Jarvis JN, Lawn SD, Vogt M, Bangani N, Wood R, Harrison TS. Screening for cryptococcal antigenemia in patients accessing an antiretroviral treatment program in South Africa. Clin Infect Dis **2009**; 48(7): 856-62.
3. Longley N, Jarvis JN, Meintjes G, et al. Cryptococcal Antigen Screening in Patients Initiating ART in South Africa: A Prospective Cohort Study. Clin Infect Dis **2016**; 62(5): 581-7.
4. Wake RM, Govender NP, Omar T, et al. Cryptococcal-related Mortality Despite Fluconazole Preemptive Treatment in a Cryptococcal Antigen Screen-and-Treat Program. Clinical Infectious Diseases **2019**; 70(8): 1683-90.
5. Tugume L, Rhein J, Hullsiek KH, et al. HIV-Associated Cryptococcal Meningitis Occurring at Relatively Higher CD4 Counts. J Infect Dis **2019**; 219(6): 877-83.
6. Yoon H, Nakouzi A, Pappas PG, Hemmige VS, Pirofski L-a. Cryptococcus neoformans-specific and non-Cryptococcous neoformans-specific antibody profiles in organ transplant recipients with and without cryptococcosis. Open Forum Infectious Diseases **2022**.
7. Hlupeni A, Nakouzi A, Wang T, et al. Antibody Responses in HIV-Infected Patients With Advanced Immunosuppression and Asymptomatic Cryptococcal Antigenemia. Open Forum Infect Dis **2019**; 6(1): ofy333.

8. Fleuridor R, Lyles RH, Pirofski L. Quantitative and qualitative differences in the serum antibody profiles of human immunodeficiency virus-infected persons with and without Cryptococcus neoformans meningitis. J Infect Dis **1999**; 180(5): 1526-35.
9. Subramaniam K, French N, Pirofski LA. Cryptococcus neoformans-reactive and total immunoglobulin profiles of human immunodeficiency virus-infected and uninfected Ugandans. Clin Diagn Lab Immunol **2005**; 12(10): 1168-76.
10. Trevijano-Contador N, Pianalto KM, Nichols CB, Zaragoza O, Alspaugh JA, Pirofski LA. Human IgM Inhibits the Formation of Titan-Like Cells in Cryptococcus neoformans. Infect Immun **2020**; 88(4).
11. Yoon HA, Nakouzi A, Chang CC, et al. Association Between Plasma Antibody Responses and Risk for Cryptococcus-Associated Immune Reconstitution Inflammatory Syndrome. J Infect Dis **2019**; 219(3): 420-8.
12. Casadevall A, Mukherjee J, Scharff MD. Monoclonal antibody based ELISAs for cryptococcal polysaccharide. Journal of immunological methods **1992**; 154(1): 27-35.
13. Deshaw M, Pirofski LA. Antibodies to the Cryptococcus neoformans capsular glucuronoxylomannan are ubiquitous in serum from HIV+ and HIV- individuals. Clin Exp Immunol **1995**; 99(3): 425-32.
14. Abadi J, Pirofski L. Antibodies reactive with the cryptococcal capsular polysaccharide glucuronoxylomannan are present in sera from children with and without human immunodeficiency virus infection. J Infect Dis **1999**; 180(3): 915-9.
15. Rohatgi S, Nakouzi A, Carreño LJ, et al. Antibody and B cell subset perturbations in HIV-uninfected patients with cryptococcosis. Open Forum Infectious Diseases **2017**: ofx255-ofx.

16. Valeri L, Vanderweele TJ. Mediation analysis allowing for exposure-mediator interactions and causal interpretation: theoretical assumptions and implementation with SAS and SPSS macros. Psychol Methods **2013**; 18(2): 137-50.
17. Li Y, Mathur MB, Yoshida K. R package regmedint: extension of regression-based causal mediation analysis with effect measure modification by covariates. OSF Preprints; Center for Open Science **2022**.
18. Hafeman DM, Schwartz S. Opening the Black Box: a motivation for the assessment of mediation. Int J Epidemiol **2009**; 38(3): 838-45.
19. Diamond RD, Bennett JE. Prognostic factors in cryptococcal meningitis. A study in 111 cases. Annals of internal medicine **1974**; 80(2): 176-81.
20. Lane HC, Masur H, Edgar LC, Whalen G, Rook AH, Fauci AS. Abnormalities of B-cell activation and immunoregulation in patients with the acquired immunodeficiency syndrome. N Engl J Med **1983**; 309(8): 453-8.
21. Houpt DC, Pfrommer GS, Young BJ, Larson TA, Kozel TR. Occurrences, immunoglobulin classes, and biological activities of antibodies in normal human serum that are reactive with Cryptococcus neoformans glucuronoxylomannan. Infect Immun **1994**; 62(7): 2857-64.
22. Zhong Z, Pirofski LA. Opsonization of Cryptococcus neoformans by human anticryptococcal glucuronoxylomannan antibodies. Infect Immun **1996**; 64(9): 3446-50.
23. Larsen RA, Pappas PG, Perfect J, et al. Phase I evaluation of the safety and pharmacokinetics of murine-derived anticryptococcal antibody 18B7 in subjects with treated cryptococcal meningitis. Antimicrob Agents Chemother **2005**; 49(3): 952-8.

24. Fleuridor R, Zhong Z, Pirofski L. A human IgM monoclonal antibody prolongs survival of mice with lethal cryptococcosis. J Infect Dis **1998**; 178(4): 1213-6.
25. Garcia-Hermoso D, Janbon G, Dromer F. Epidemiological evidence for dormant Cryptococcus neoformans infection. J Clin Microbiol **1999**; 37(10): 3204-9.
26. Zhang MX, Bohlman MC, Itatani C, et al. Human Recombinant Antimannan Immunoglobulin G1 Antibody Confers Resistance to Hematogenously Disseminated Candidiasis in Mice. Infection and Immunity **2006**; 74(1): 362-9.
27. Clancy CJ, Nguyen ML, Cheng S, et al. Immunoglobulin G responses to a panel of Candida albicans antigens as accurate and early markers for the presence of systemic candidiasis. J Clin Microbiol **2008**; 46(5): 1647-54.
28. Maddur MS, Lacroix-Desmazes S, Dimitrov JD, Kazatchkine MD, Bayry J, Kaveri SV. Natural Antibodies: from First-Line Defense Against Pathogens to Perpetual Immune Homeostasis. Clin Rev Allergy Immunol **2020**; 58(2): 213-28.
29. Wake RM, Britz E, Sriruttan C, et al. High Cryptococcal Antigen Titers in Blood Are Predictive of Subclinical Cryptococcal Meningitis Among Human Immunodeficiency Virus-Infected Patients. Clin Infect Dis **2018**; 66(5): 686-92.
30. Rajasingham R, Wake RM, Beyene T, Katende A, Letang E, Boulware DR. Cryptococcal Meningitis Diagnostics and Screening in the Era of Point-of-Care Laboratory Testing. J Clin Microbiol **2019**; 57(1).
31. Rodrigues ML, Travassos LR, Miranda KR, et al. Human antibodies against a purified glucosylceramide from Cryptococcus neoformans inhibit cell budding and fungal growth. Infect Immun **2000**; 68(12): 7049-60.

32. Rachini A, Pietrella D, Lupo P, et al. An anti-beta-glucan monoclonal antibody inhibits growth and capsule formation of Cryptococcus neoformans in vitro and exerts therapeutic, anticryptococcal activity in vivo. Infect Immun **2007**; 75(11): 5085-94.
33. Torosantucci A, Chiani P, Bromuro C, et al. Protection by anti-beta-glucan antibodies is associated with restricted beta-1,3 glucan binding specificity and inhibition of fungal growth and adherence. PLoS One **2009**; 4(4): e5392.
34. Torosantucci A, Tumbarello M, Bromuro C, et al. Antibodies against a beta-glucan-protein complex of Candida albicans and its potential as indicator of protective immunity in candidemic patients. Sci Rep **2017**; 7(1): 2722.
35. Schwebach JR, Glatman-Freedman A, Gunther-Cummins L, et al. Glucan Is a Component of the Mycobacterium tuberculosis Surface That Is Expressed In Vitro and In Vivo. Infection and Immunity **2002**; 70(5): 2566-75.
36. Costello AM, Kumar A, Narayan V, et al. Does antibody to mycobacterial antigens, including lipoarabinomannan, limit dissemination in childhood tuberculosis? Trans R Soc Trop Med Hyg **1992**; 86(6): 686-92.
37. Jarvis JN, Harrison TS, Corbett EL, Wood R, Lawn SD. Is HIV-associated tuberculosis a risk factor for the development of cryptococcal disease? Aids **2010**; 24(4): 612-4.
38. Wake RM, Ismail NA, Omar SV, et al. Prior Pulmonary Tuberculosis is a Risk Factor for Asymptomatic Cryptococcal Antigenemia in a Cohort of Adults living with Advanced HIV Disease. Open Forum Infectious Diseases **2022**.
39. Subramaniam K, Metzger B, Hanau LH, et al. IgM(+) memory B cell expression predicts HIV-associated cryptococcosis status. J Infect Dis **2009**; 200(2): 244-51.

40. Nanfuka V, Mkhoi ML, Gakuru J, et al. Symptomatic Cryptococcal Meningitis with Negative Serum and Cerebrospinal Fluid Cryptococcal Antigen Tests. HIV AIDS (Auckl) **2021**; 13: 861-5.

Table 1. Baseline and clinical characteristics of participants by CrAg status

Covariables	Total (n=197)	CrAg-negative (n=130)	CrAg-positive (n=67)	*P*-value
Age, year	39.0 (32.4-47.1)	39.1 (33.0-47.8)	38.7 (31.7-47.1)	0.67
Sex				
Male	100 (50.8)	70 (53.9)	30 (44.8)	0.23
Female	97 (49.2)	60 (46.2)	37 (55.2)	
Race				
Black	191 (97.0)	125 (96.2)	66 (98.5)	0.67
Others	6 (3.1)	5 (3.9)	1 (1.5)	
CD4 count (cells/mm^3)	33 (12-61)	40.5 (16-64)	27 (7-40)	**0.003**
Plasma CrAg titers	1:40 (5-320)	n/a	1:40 (5-320)	
Baseline blood fungal growth	5 (2.5)	0	5 (7.5)	**0.002**
Tuberculosis in the past 6 mo.[1]	51 (25.9)	33 (25.4)	18 (26.9)	0.82
Tuberculosis treatment	29 (14.7)	18 (13.9)	11 (16.4)	0.63
ART prior to enrollment	29 (14.7)	13 (10.0)	16 (23.9)	**0.01**
Vital status at 6 mo.				
Alive	160 (81.2)	113 (86.9)	47 (70.2)	**0.01**
Dead	28 (14.2)	11 (8.5)	17 (25.4)	

Unknown	9 (4.6)	6 (4.6)	3 (4.5)

Note: Categorical variables are summarized with counts, percentages and *P*-values based on Fisher's exact or χ^2 test. Continuous variables are summarized with medians, interquartile range and *P*-values based on Wilcoxon rank-sum test. *P*-values < 0.05 bolded.

[1]Includes pulmonary, disseminated, or extrapulmonary *Mycobacterium tuberculosis* infection diagnosed within 6 months prior to, or on the day of enrollment.

Abbreviations: ART, antiretroviral therapy; CrAg, cryptococcal antigen; HIV, human immunodeficiency virus; mo, months; n/a, not applicable.

Table 2. Stratified Cox proportional hazards regression model estimating GXM-IgG as a predictor of mortality by 6 months following enrollment.

	CrAg-negative (n=130)		**CrAg-positive (n=67)**	
	HR (95% CI)	***P*-value**	**HR (95% CI)**	***P*-value**
Death, no. (%)	11 (8.5)		17 (25.4)	
Model 1 – Unadjusted	1.03 (0.58-1.80)	0.93	0.55 (0.36-0.84)	**0.006**
Model 2 – Adjusted CD4 count	1.04 (0.59-1.82)	0.90	0.56 (0.36-0.86)	**0.009**
Model 3 – Adjusted CD4 count and tuberculosis	1.03 (0.60-1.76)	0.92	0.50 (0.33-0.77)	**0.002**

Note: GXM-IgG was log-transformed and used as a continuous variable. *P*-values < 0.05 bolded.

Abbreviations: CI, confidence interval; CrAg, cryptococcal antigen; GXM, glucuronoxylomannan; HR, hazard ratio.

Figure legends

Figure 1. Total and antigen-specific antibody levels in 130 CrAg-negative and 67 CrAg-positive individuals at baseline. Total Ig levels were determined by Luminex and GXM, laminarin, and curdlan antibody titers were determined by enzyme-linked immunosorbent assay. Ig concentrations in units of μg/ml (A) and the inverse of GXM (B), laminarin (C), and curdlan (D) titers, depicted as medians and interquartile ranges, are shown on the y-axis for each group shown on the x-axis. CrAg status is represented by (+) or (-) to indicate positive or negative CrAg status, respectively. *P<0.05; **P≤0.001. Wilcoxon rank-sum test. Abbreviation: CrAg, cryptococcal antigen; GXM, glucuronoxylomannan; Ig, immunoglobulin.

Figure 2. Forest plots of the univariable (A) and multivariable (B) logistic regression analysis estimating the association between antibody markers and plasma CrAg status in a cohort of 130 CrAg-negative and 67 CrAg-positive individuals. Antibody variables were log-transformed. Covariables with P<0.20 in the univariable analysis were included in the multivariable analysis (CD4 count and ART use). Tuberculosis includes pulmonary, disseminated, or extrapulmonary infection diagnosed within 6 months prior to enrollment. ART use includes treatment history prior to enrollment. P-values < 0.05 bolded. Abbreviations: aOR, adjusted odds ratio; ART, antiretroviral therapy; CI, confidence interval; CrAg, cryptococcal antigen; GXM, glucuronoxylomannan; OR, odds ratio.

Figure 3. Kaplan-Meier Curves for survival by baseline GXM-IgG categories. The range of inverse titers of GXM-IgG was categorized into tertiles; low (1.9-62.2), medium (62.8-172.0), and high GXM-IgG (176.3-3,453.3). Curves are shown for the 67 CrAg-positive individuals

(Panel A) and 130 CrAg-negative individuals (Panel B). GXM-IgG was log-transformed in the survival analysis. Abbreviations: GXM, glucuronoxylomannan.

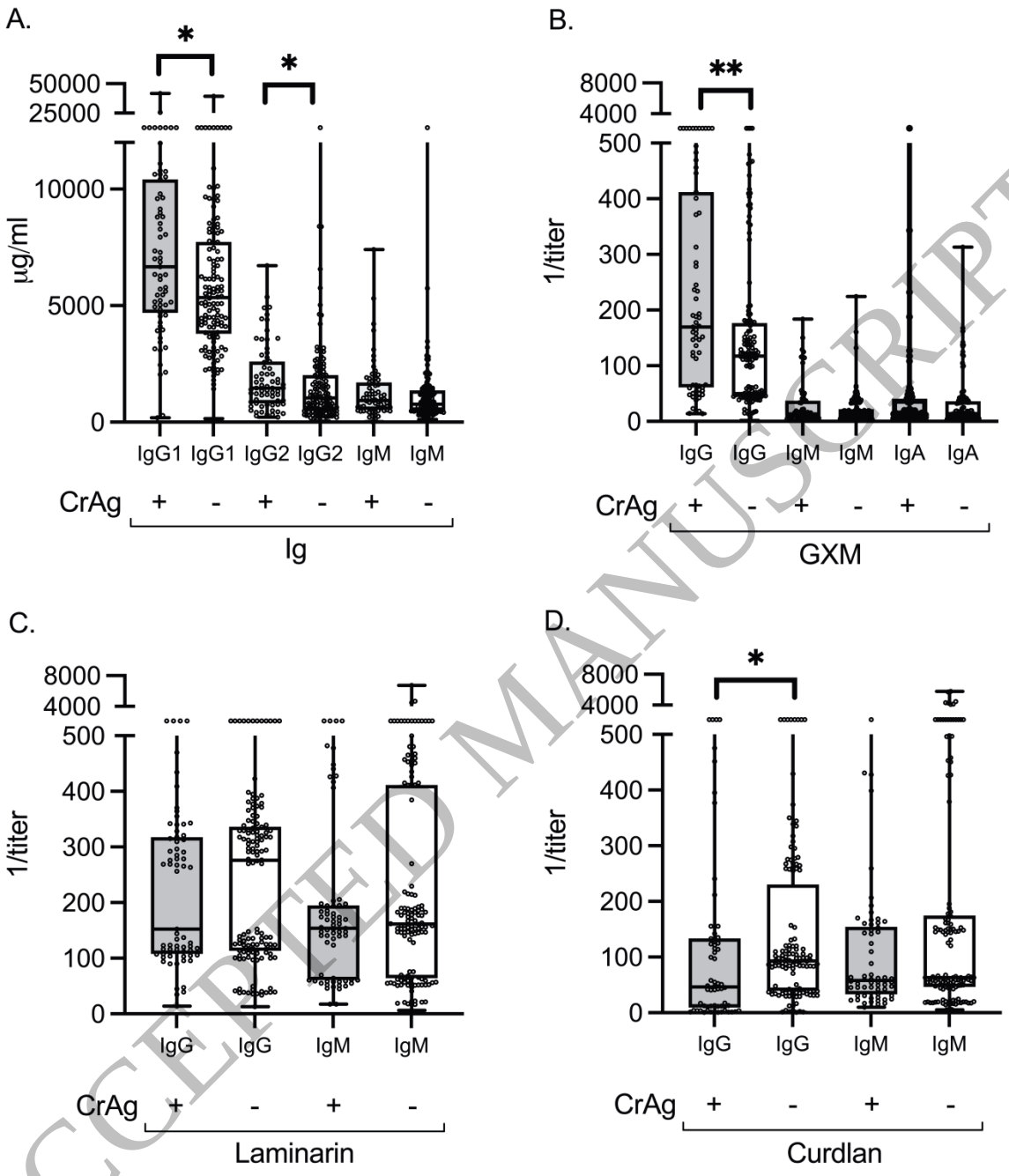

Figure 1
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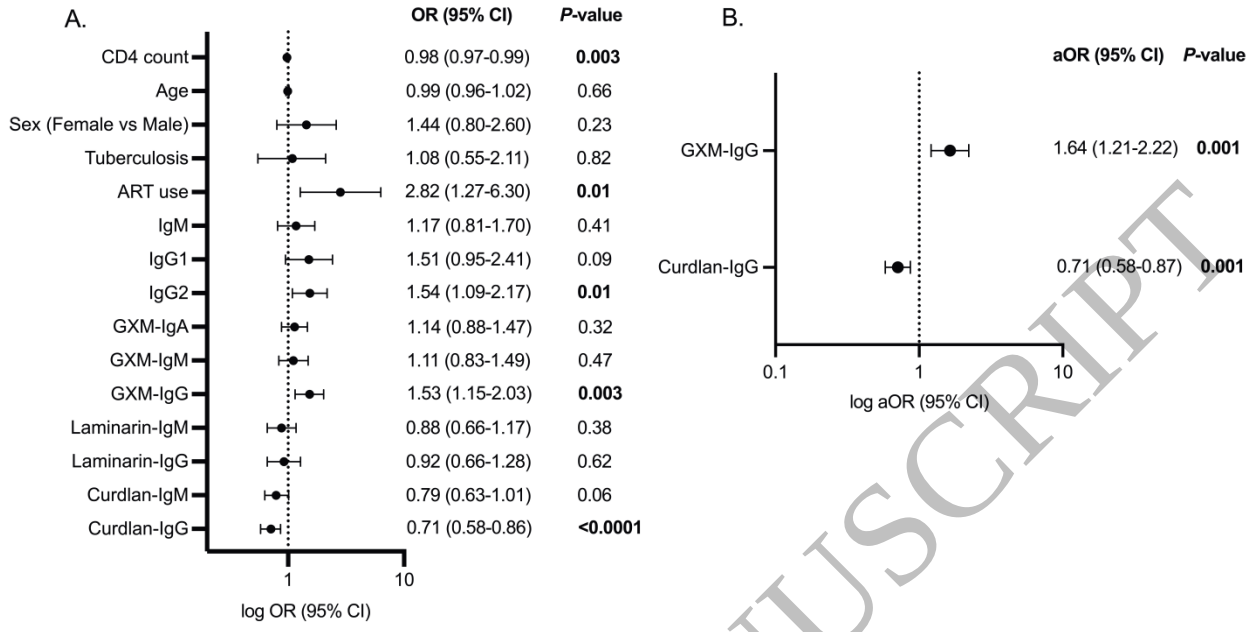

Figure 2
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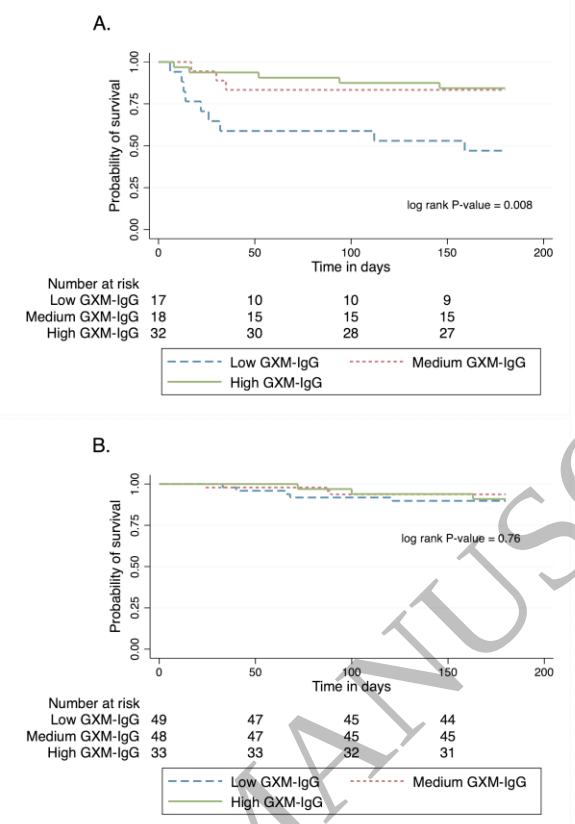

Figure 3
76x110 mm (x DPI)