

Antibody Responses in HIV-Infected Patients With Advanced Immunosuppression and Asymptomatic Cryptococcal Antigenemia

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Background. There are no host biomarkers of risk for HIV-associated cryptococcal meningitis (CM) except CD4+ T-cell deficiency. At present, serum cryptococcal antigen (CrAg) screening of those with CD4 <100 cells/μL is used to identify persons at risk for HIV-associated CM. We determined if plasma antibody profiles could discriminate CrAg+ from CrAg- patients.

Methods. We performed serological analyses of 237 HIV-infected asymptomatic Zimbabwean patients with CD4 <100 cells/μL; 125 CrAg- and CrAg+ but cerebrospinal fluid CrAg- by CrAg lateral flow assay. We measured plasma immunoglobulin M (IgM), immunoglobulin G (IgG) 1, and IgG2 concentrations by Luminex, and titers of *Cryptococcus neoformans* (Cn) glucuronoxylomannan (GXM) polysaccharide and naturally occurring Laminarin (natural Lam, a β-(1-3)-glucan linked polysaccharide)-binding IgM and IgG by enzyme-linked immunosorbent assay.

Results. GXM-IgG, -IgM, and -IgG2 levels were significantly higher in CrAg+ patients, whereas natural Lam-IgM and Lam-IgG were higher in CrAg- patients before and after adjustment for age, sex, and CD4 T-cell count, despite overlap of values. To address this variability and better discriminate the groups, we used Akaike Information Criteria to select variables that independently predicted CrAg+ status and included them in a receiver operating characteristic curve to predict CrAg status. By inclusion of CD4, GXM-IgG, GXM-IgM, and Lam-IgG, -IgG2, and -IgM, this model had an 80.4% probability (95% confidence interval, 0.75–0.86) of predicting CrAg+ status.

Conclusions. Statistical models that include multiple serological variables may improve the identification of patients at risk for CM and inform new directions in research on the complex role that antibodies may play in resistance and susceptibility to CM.

Keywords. antibody; cryptococcal antigenemia; glucuronoxylomannan; HIV; IgG; IgM; immunoglobulin; Laminarin; Sub-Saharan Africa; Zimbabwe.

Cryptococcus neoformans (Cn) is the leading cause of meningitis among individuals living with HIV [1]. Using the 2014 Joint UN Programme on HIV and AIDS estimates, annual global deaths from cryptococcal meningitis (CM) were 181 100, with 75% of the deaths occurring in sub-Saharan Africa (SSA) [2]. Despite increased access to HIV testing and antiretroviral therapy (ART) in SSA, the incidence of CM is largely unchanged [3]. Notably, 40% of patients with CM in Southeast Asia and SSA were on ART at the time of presentation, many for several months [4].

There are no validated host biomarkers of risk for CM, except CD4 T-cell deficiency. In SSA and elsewhere, the risk of HIV-associated CM is assessed by serum cryptococcal antigen (CrAg) testing of those with ≤100 CD4 T cells/μL [5]. Although this is cost-effective [6], HIV-associated CM also occurs in patients with CD4 T-cell counts ≥100 cells/μL [7], and most HIV-uninfected patients with CM have CD4 T cells in the normal range [8]. Therefore, serological biomarkers of risk for CM could cast a wider net and enable earlier risk stratification. Biomarkers of CM may also provide new insight into host factors that promote dissemination of Cn and inform development of immune-based therapy and vaccines.

HIV-infected (HIV+) persons with advanced immunosuppression and CD4 T-cell deficiency are at the highest risk for reactivating latent Cn, but not all do so. The role of cell-mediated immunity in resistance to Cn in animals and humans is well known, but antibody is also important [9, 10]. In mice, the absence of B-1 cells and/or natural immunoglobulin M (IgM) increases Cn dissemination to the brain [11–13]. Defined cryptococcal glucuronoxylomannan (GXM) capsular

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polysaccharide-specific immunoglobulin G (IgG) monoclonal antibodies (Mabs) protect mice against lethal Cn challenge [9], but high doses can fail to do so, as GXM-IgG-Cn immune complexes can inhibit phagocytosis and other host defense mechanisms [14]. A similar phenomenon is plausible in humans, particularly those who express the FcγRIII allele (V158), which binds serum IgG-GXM complexes with higher affinity than a low-affinity allele (F158) and is associated with CM in HIV+ and HIV-uninfected (HIV-) persons [15, 16].

In numerous studies, HIV+ and HIV- patients with CM and HIV+ persons had higher levels of GXM-IgG than, respectively, those without CM and HIV- persons [8, 17, 18]. On the other hand, GXM-IgM was lower in patients with than without HIV-associated CM and solid organ transplant recipients who developed post-transplant CM compared with those who did not [15, 18–20]. Nonetheless, GXM IgM and IgG levels can be variable and are not sufficient to identify patients who are at risk for CM. Therefore, we sought to determine if multiple antibody variables could better identify HIV+ patients at risk for CM. To test this idea, in addition to GXM-binding antibodies, we measured levels of plasma immunoglobulins and naturally occurring Laminarin (Lam)-binding antibodies in asymptomatic cryptococcal antigen-positive (CrAg+) and -negative (CrAg-) HIV-infected patients. HIV infection has a profound effect on B-cell activation and immunoglobulin levels [21, 22]. Antibodies that bind Lam, a mainly 1–3-β glucan-linked polysaccharide, bind fungi, including Cn [23, 24] and are part of the natural human antibody repertoire [25].

METHODS

Ethical Approvals

This study was approved by the Joint Research Ethics Committee of the University of Zimbabwe College of Health Sciences and Parirenyatwa Group Hospitals (JREC/113/15), the Medical Research Council of Zimbabwe (MRCZ/B/881), and the Institutional Research Board (IRB) at the Albert Einstein College of Medicine (Bronx, NY). Sample storage and specimen shipment were approved by the Research Council of Zimbabwe (RCZ). All participants included in this substudy had provided written informed consent for sample storage, future use, and shipment in the parent CryptoART study.

Study Population

This is a substudy of the CryptoART study (ClinicalTrials.gov Identifier: NCT02434172). The cohort consisted of banked plasma samples from HIV+ persons originally recruited into the CryptoART study in Harare, Zimbabwe, between April 2015 and June 2016. The study was a 12-month prospective implementation science study in which serum CrAg testing by CrAg lateral flow assay (LFA) was conducted in HIV-1-infected individuals (age ≥18 years) with CD4 T-cell counts <100 cells/

μL receiving care at outpatient treatment care facilities in Harare, Zimbabwe. Eligible participants were required to have no clinical symptoms suggestive of meningitis. A screening questionnaire excluded participants with symptoms suggestive of meningitis or focal neurological signs that included a headache, confusion, stiff neck, vision changes, seizures, altered behavior, and focal weakness. Participants were excluded if they had a recent history of CM within 2 weeks of screening, previous allergy or other reaction to amphotericin B and/or fluconazole, an estimated glomerular filtration rate (eGFR) of ≤30 mL/min, or alanine transaminase ≥5 times the upper limit of normal. Pregnant women were excluded. None of the participants included in this substudy were on antiretroviral therapy, and none had a history of CM or evidence of disseminated cryptococcosis, as defined by a positive blood or cerebrospinal fluid (CSF) fungal culture.

The samples used in this substudy included a subset of serum CrAg- and CrAg+ but CSF CrAg- patients matched by age, sex, and CD4 T-cell count who did not have clinical or laboratory evidence of cryptococcal dissemination (Supplementary Table 1).

Measurement of GXM and Natural Lam Antibody Titers

Plasma GXM-IgM, GXM-IgG, and Lam-IgM and -IgG titers were determined by enzyme-linked immunosorbent assay as previously described [26]. Lam is a branched chain polysaccharide composed mainly of 1–3-β glucan linkages [24]; β glucans are part of the Cn cell wall [27, 28]. Plasma samples were used at an initial 1:10 dilution and serially diluted in a 96-well Costar plate (Corning Inc., Kennebunk, ME) coated with 10 μg/mL of GXM (from CN 24067) or Lam (Sigma-Aldrich). Plates were incubated with plasma, washed, and incubated with isotype-specific goat antihuman alkaline phosphatase labeled Abs for 1 hour (GAH IgM-AP, GAH IgG-AP; Southern Biotech, Birmingham, AL) and then *p*-nitrophenyl phosphatase (Thermo Scientific, Rockford, IL), after which the absorbance at 405 nm was measured with a VERSAmax plate reader (Molecular Devices, Sunnyvale, CA). Titers were defined as the absorbance (405 nm) of the highest dilution to give a signal ≥2 times the background (wells without serum) with the dilution factor.

Measurement of Immunoglobulin Levels

Total immunoglobulin levels of IgM, IgG1, and IgG2 in plasma samples were determined by Luminex as previously described [26] using a MILLIPLEX MAP kit (human immunoglobulin isotyping magnetic beads; EMD Millipore Corp., Billerica, MA) according to the manufacturer's instructions.

Statistical Analysis

Serological results were compared between the CrAg+ and CrAg- groups using the Wilcoxon rank test for significance.

Spearman correlations were used to seek associations between GXM-IgG levels and CrAg titers. The log-transformed serological results were further compared using linear regression models with adjustment for age, sex, and log-CD4 count. *P* values of <.05 were considered statistically significant. To identify serological variables that can predict CrAg status, we performed a stepwise model selection using the Akaike Information Criterion (AIC) [29] in a logistic regression model with age, sex, log-CD4, and all log-transformed serological results. We assessed the discriminatory accuracy of the selected model by the receiver operating characteristic (ROC) curve (R, version 2.3, and R library pROC, R Development Core Team, <http://www.R-project.org>) and the corresponding area under the curve (AUC). To evaluate the contribution of each variable in the model, we compared the AUCs before and after removing each variable from the selected models. ROC curves plot the true-positive rate (sensitivity) vs the false-positive rate (1 - specificity) at a continuum of thresholds; this predicts a participant's CrAg status if the estimated probability exceeds a particular threshold. An ROC curve of a nonpredictive model is a straight line with an AUC of 0.50.

RESULTS

We measured antibody levels in banked plasma samples from 237 HIV+ participants from the parent CryptoART study. This included 125 participants who were serum CrAg- and 112 who were serum CrAg+ but CSF CrAg-, with a median (interquartile range) serum CrAg titer of 1:20 (1:5–1:80). The baseline demographic and clinical characteristics of the CrAg+ and CrAg- groups were not statistically significantly different (Supplementary Table 1).

Plasma Antibody Levels

GXM Antibodies

GXM-IgG titers were significantly higher in CrAg+ than CrAg- participants before and after adjustment for age, sex, and CD4 (Figure 1A, Table 1). GXM-IgM titers were significantly lower in CrAg+ than CrAg- participants, but statistical significance was attenuated (*P* = .08) after adjustment for age, sex, and CD4 (Figure 1A, Table 1).

We also determined GXM-IgG1 and GXM-IgG2 levels of a subset of 10 CrAg+ and 10 CrAg- participants with the highest GXM-IgG levels and a subset of 9 CrAg+ and 10 CrAg- participants with low GXM-IgG levels, as described previously [17, 30]. We excluded 1 CrAg+ sample from this and all other data sets because the patient was CSF CrAg+. GXM-IgG1 titers were statistically significantly higher in CrAg+ participants with high GXM-IgG titers, despite a range of values (Supplementary Figure 1A). There were no differences in the GXM-IgG2 titers of CrAg+ and CrAg- participants with low GXM-IgG titers (Supplementary Figure 1B). There was no significant correlation between CrAg titer and GXM-IgG, GXM-IgG1, or GXM-IgG2 levels (Supplementary Table 1). However, for the subset of participants with high GXM-IgG, the Spearman coefficient for the correlation between CrAg titer and GXM-IgG1 was 0.48, but at *P* = 0.19, it did not reach statistical significance, perhaps because of the small sample size.

Lam Antibodies

Lam-IgM and Lam-IgG titers were significantly lower in CrAg+ than CrAg- participants before and after adjustment for age, sex, and CD4 (Figure 1B, Table 2).

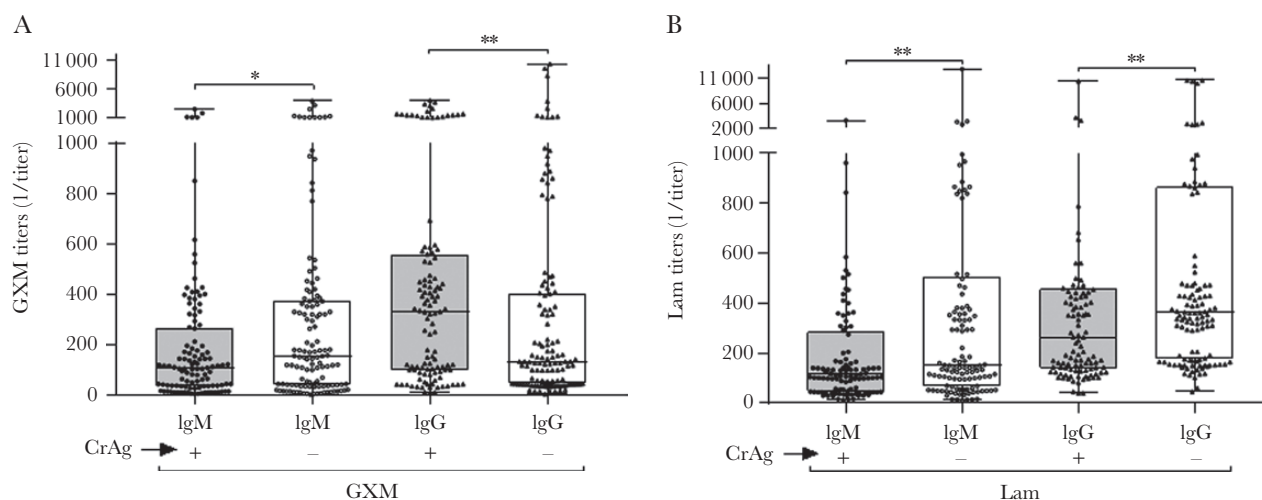


Figure 1. Glucuronoxylomannan (GXM) antibody titers determined by enzyme-linked immunosorbent assay. The inverse of (A) GXM-immunoglobulin M (IgM) and GXM-immunoglobulin G (IgG) and (B) Laminarin (Lam)-IgM and Lam-IgG titers, depicted as medians and interquartile ranges, are shown on the y-axis for each group shown on the x-axis. Cryptococcal antigen (CrAg) status is represented by (+) or (-) to indicate positive or negative CrAg status, respectively. **P* < .05; ***P* ≤ .001, Wilcoxon rank-sum test.

Table 1. Associations of Plasma GXM, Lam, and Immunoglobulin Levels With CrAg Status

	Univariate Analysis ^a			Multiple Regression ^b	
	CrAg+, Median (IQR)	CrAg-, Median (IQR)	PValue	Coefficient	PValue
IgM	951 (1066)	785 (408)	.001	.296	0
IgG1	8165 (6275)	7246 (5498)	.074	.048	.525
IgG2	2280 (2252)	1281 (1733)	1.3e-5	.598	.001
GXM-IgM	110 (229)	155 (332)	.044	-.335	.08
GXM-IgG	331 (448)	131 (357)	.006	.448	.012
Lam-IgM	115 (229)	153 (428)	.006	-.448	.005
Lam-IgG	265 (317)	364 (689)	.004	-.39	.003

Abbreviations: CrAg, cryptococcal antigen; GXM, glucuronoxylomannan; IgG, immunoglobulin G; IgM, immunoglobulin M; IQR, interquartile range; Lam, Laminarin.

^aWilcoxon rank test.

^bRegression: log-transformed levels of antibodies compared between CrAg+ and CrAg- participants with adjustment for age, sex, and logCD4

Plasma Immunoglobulins

The concentrations of IgM and IgG2 were significantly higher in the plasma of CrAg+ than CrAg- participants before and after adjustment for age, sex, and CD4 (Figure 2, Table 1).

Associations of Antibody Levels With CrAg Status

IgM, IgG2, and GXM-IgG were each independently positively associated with CrAg+ status, whereas Lam-IgG was negatively associated with CrAg+ status (Table 1). Use of a backward variable selection based on AIC [29] to identify markers that may predict CrAg+ status (Table 2) resulted in an AUC of 80.4% (95% confidence interval [CI], 0.75–0.86) when GXM-IgG, Lam-IgG, IgG2, IgM, CD4, and GXM-IgM were included in the model (Figure 3). Thus, this model had an 80% probability to predict CrAg+ status. After individually removing these variables from the model, CD4 and GXM-IgM did not affect the AUC, but the AUC was lower when we removed GXM-IgG, Lam-IgG, IgG2, and IgM (Figure 3). As a comparison, the model with age, sex,

Table 2. Logistic Regression to Predict Positive CrAg Identified by a Backward Variable Selection Approach Using Akaike Information Criteria

Variable	Estimate	SE	Z Value	PValue
(Intercept)	-5.319792	3.213608	-1.655	.09784
Age	-0.005644	0.020427	-0.276	.78233
SexM	-0.011674	0.326033	-0.036	.97144
Log_CD4	-0.270405	0.192485	-1.405	.16008
Log_GXM-IgM	-0.192239	0.153771	-1.250	.21124
Log_GXM-gG	0.698657	0.159256	4.387	1.15e-05
Log_Lam-IgM	-0.192441	0.202436	-0.951	.34179
Log_Lam-IgG	-0.978921	0.235184	-4.162	3.15e-05
Log_IgG1	-0.190509	0.313837	-0.607	.54383
Log_IgG2	0.358727	0.128147	2.799	.00512
Log_IgM	1.387287	0.328529	4.223	2.41e-05

Abbreviations: CrAg, cryptococcal antigen; GXM, glucuronoxylomannan; IgG, immunoglobulin G; IgM, immunoglobulin M; Lam, Laminarin.

and CD4 did not have any prediction ability with an AUC of 0.53 (95% CI, 0.46–0.60).

DISCUSSION

CD4 T-cell counts <100 cells/μL are an important risk factor for HIV-associated CM but are insufficient to predict which patients will develop HIV-associated CM. To seek serological biomarkers of risk, we measured Cn GXM capsule-binding and natural 1–3-β-glucan-binding antibody and immunoglobulin levels in plasma samples from asymptomatic HIV-infected CrAg+ and CrAg- individuals with <100 CD4 T cells. We found that CrAg+ participants had statistically significantly higher levels of GXM-IgG, IgG2, and IgM and lower levels of Lam-IgM and IgG than CrAg- participants, which remained significant after adjustment for age, sex, and CD4 T-cell count. Like many biological variables, antibody levels of CrAg+ and CrAg- participants overlapped. To improve our ability to differentiate the groups, we developed a prediction model that included multiple antibody variables. The model selected 6 variables, GXM-IgG, Lam-IgG, IgG2, IgM, GXM-IgM, and CD4, for inclusion in an ROC curve that had an 80% ability to predict CrAg+ status, however, removal of GXM-IgM and CD4 did not affect the model. As single host factors are rarely able to predict susceptibility to many if not most diseases, our data suggest that statistical modeling is a promising tool to identify host biomarkers of risk for complex diseases like CM.

GXM-IgG titers were higher in the plasma of CrAg+ than CrAg- participants. This parallels previous reports in which GXM-IgG was higher in HIV+ and HIV- patients with CM than without CM [8, 17, 18]. There is ample evidence that serum CrAg heralds symptomatic CM [31], and GXM-IgG was an independent predictor of CrAg+ status in our cohort. However, we did not find a correlation between GXM-IgG and CrAg titer, perhaps due to the small sample size or because CrAg does not faithfully reflect fungal burden. Soluble GXM can form complexes with GXM-IgG, but dissociation of such complexes did not affect measurements in a recent study [8].

Although descriptive, our data suggest that certain mechanistic effects of GXM-IgG identified in mice may be plausible in humans. For example, mouse GXM-IgG protected mice against lethal Cn, but high doses interfered with host defense in the setting of high inocula and abrogated protection [14]. GXM-IgG can promote intracellular uptake without killing [32], which can facilitate Cn transport to the brain in infected phagocytic cells, as described for Cn-infected human mononuclear cells in mice [33]. A similar scenario is plausible in humans given that Cn-human IgG immune complexes exhibited enhanced binding to and triggered more of a cytotoxic response upon binding with the high-affinity Fc-gamma-RIII allele V158 [15]. Expression of the V158 allele has been associated with CM in HIV+ and HIV- individuals [15, 16].

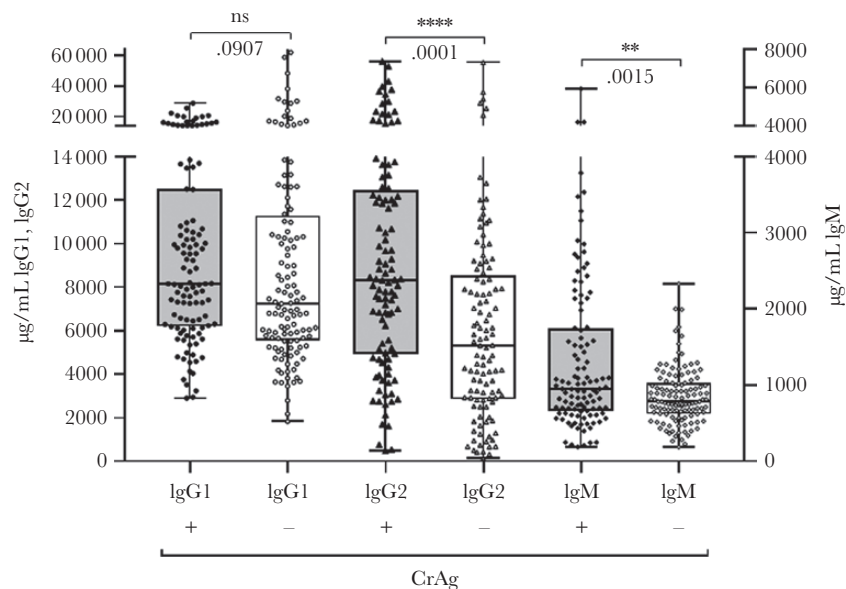


Figure 2. Total immunoglobulin levels determined by Luminex. Levels of immunoglobulin M (IgM), immunoglobulin G (IgG) 1, and IgG2, depicted as medians with interquartile ranges, are shown on the left (IgG1) and right (IgG2, IgM) y-axis for each isotype and group shown on the x-axis. Cryptococcal antigen (CrAg) status is represented by (+) or (-) to indicate positive or negative CrAg status, respectively. * $P \leq .001$; **** $P \leq .0001$, Wilcoxon rank-sum test. Abbreviation: ns, not significant.

Plasma Lam-IgM and Lam-IgG were lower in CrAg+ than CrAg- participants and were inversely associated with CrAg+ status, although only the association with Lam-IgG was significant after adjustment for age, sex, and CD4. Lam-IgG was

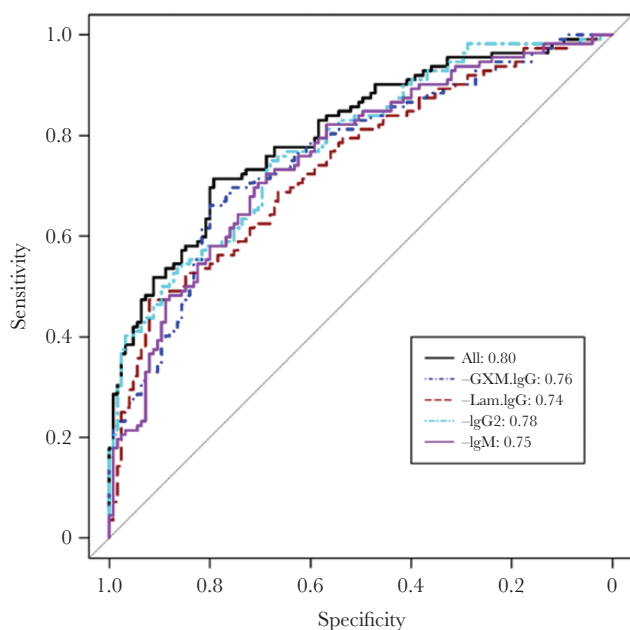


Figure 3. Receiver operating characteristic (ROC) curve. The ROC curve for all selected variables (black line) compared with models after removal of each of the prediction variables in the model indicated in the box (dark blue dashed line, glucuronoxylomannan [GXM]-immunoglobulin G [IgG]; brown dashed line, Laminarin [Lam]-IgG; light blue dashed line, IgG2; pink solid line, immunoglobulin M [IgM]). The area under the ROC curve with all selected variables is 0.804 (95% confidence interval, 0.748–0.859).

also independently predictive of CrAg- status. Lam-binding antibodies mediate protection in mice against multiple fungi, including Cn [23, 24, 27] via binding to cell wall 1-3- β glucans. Given that Lam-binding antibodies are part of the natural pre-immune human serological repertoire [25] and bind to 1-3- β glucans [25], it is reasonable to posit they may play a beneficial role in protection against human CM. In support of this idea, a Lam-IgG monoclonal antibody inhibited Cn growth in vitro and protected mice against a lethal Cn challenge [23], and human Cn cell wall 1-3- β -glycosylceramide-binding IgG inhibited Cn growth in vitro [34]. Lam-IgM and IgG were also lower in HIV+ patients with cryptococcal immune reconstitution inflammatory syndrome (C-IRIS) than without C-IRIS [26]. Neither the aforementioned nor this study included HIV- persons or mechanistic studies. Nonetheless, our findings suggest the hypothesis that human antibodies may enhance resistance to CM and worthy of investigation that bind fungal beta-glucans.

Hypergammaglobulinemia is a hallmark of advanced HIV infection [21], including in African patients [22]. Although IgG2 is often lower in HIV+ than HIV- Caucasians [35], IgG2 and IgM were each elevated in HIV+ Nigerians [36]. We found that plasma IgM and IgG2 were higher in CrAg+ than CrAg- participants and were independently associated with CrAg+ status. In CrAg+ participants, IgM and IgG2 levels may reflect B-cell activation by Cn. IgM memory B cells produce naturally occurring antibodies, and IgG2 is the main IgG subclass of human GXM antibodies [26, 30, 37]. In experimental models, IgM conferred protection against Cn dissemination in mice [11, 13], and mouse GXM-human-IgG2 chimeric Mabs protected

mice against lethal Cn, but chimeric IgG1 was not protective [38]. Notably, IgG1 did not differ between CrAg+ and CrAg- participants in our cohort. In humans, serum IgG2, not IgG1, from volunteer recipients of an experimental GXM conjugate vaccine was the main mediator of human mononuclear cell phagocytosis of Cn [30]. We were not able to measure GXM-IgG1 or GXM-IgG2 in our entire cohort, but GXM-IgG1 titers of a subset of CrAg+ participants with the highest GXM-IgG titers were statistically significantly higher than those of CrAg- participants. This finding pertains to a small subset of high-titer GXM-IgG samples and requires validation in a larger cohort. Nonetheless, it suggests that production of GXM-IgG1 may be associated with risk for CM, because it lacks functional efficacy against Cn. This hypothesis warrants investigation as efficacy of mouse GXM-IgG Mabs was a function of IgG subclass [9].

In summary, our data show significant differences in levels of IgG2, IgM, and GXM and natural Lam antibodies in the plasma of asymptomatic HIV+ ART-naïve CrAg+ and CrAg- individuals in SSA. Our study has several limitations; we did not have an HIV- control group, we were not able to perform IgG subclass analyses or mechanistic experiments, and the participants had advanced immunosuppression that may have affected serological variables. Nonetheless, we were able to construct a model with an 80% probability of predicting CrAg+ status in our cohort. We hope to test this model in other cohorts of HIV+ persons. If validated, the availability of a multiple antibody detection platform could improve upon current CrAg screening for CM in SSA, where the scarcity of amphotericin B contributes to treatment failure and mortality. In addition, our data suggest that basic scientific studies of the role that GXM and beta-glucan-(natural Lam) binding antibodies and IgG subclasses play in the pathogenesis of human CM could bring a deeper understanding of resistance and susceptibility to this disease. Finally, validation of the model that emerged from this study could pave the way for improved identification of other patients at risk for CM, for example, HIV+ patients with higher CD4 counts, solid organ transplant recipients, patients with other immunodeficiency states [39], and patients on biologics [40].

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Acknowledgments

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Trial registration. The parent study is registered at ClinicalTrials.gov (Identifier: NCT00830856).

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Potential conflicts of interest. All authors: no reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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