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Cryptococcus neoformans- and *Cryptococcus gattii*-specific IgG, IgA and IgM differ among children and adults with and without cryptococcosis from Colombia

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Abstract

Cryptococcus neoformans (*Cn*) and *Cryptococcus gattii* (*Cg*) cause cryptococcosis, a life-threatening systemic mycosis of global distribution affecting mainly immunocompromised adults. Although a humoral response occurs during cryptococcosis, the role of antibody production against this mycosis is not fully understood. We aimed to determine total and specific antibodies against cryptococcal protein antigens in sera from people with and without a diagnosis of cryptococcosis from Colombia. Using ELISA, total and specific levels of immunoglobulin (Ig)G, IgA and IgM were determined in sera from children and adults with ($n = 109$) and without ($n = 119$) cryptococcosis. Specific antibodies were those binding *Cn*- and *Cg*-protein antigens. In general, the mean of the total IgG production was higher in cryptococcosis patients than in controls (13 942.32 vs. 6459.91 µg/ml), while levels of IgA (488.13 vs. 1564.53 µg/ml) and IgM (775.69 vs. 1014.72 µg/ml) were higher in controls than in cryptococcosis patients ($P \leq .05$). In patients with cryptococcosis, total IgG, IgA and IgM levels were higher in HIV + compared with HIV− ($P \leq .05$). Specific antibodies tended to be higher in cryptococcosis patients than in controls and in adults than in children, with a positive correlation between antibody reactivity and age. All immunoglobulins were more reactive against *Cn*-proteins than *Cg*-proteins. Overall, a positive weak correlation between total and specific antibodies was found, although not always statistically significant. In patients with cryptococcosis from Colombia, the levels of immunoglobulins, total and specific, differ with respect to people without cryptococcosis. Variations in antibody production among adults and children with cryptococcosis and between *Cn*- and *Cg*-protein antigens were as well established. Our findings encourage further studies to determine the role of humoral immunity for host defense against cryptococcosis.

Lay Summary

Differential IgG, IgA, and IgM production and their reactivity with cryptococcal proteins, both among children and adults with and without a diagnosis of cryptococcosis from Colombia, lead to reappraise the study of the potential role of antibody production as host defense against this fungal infection.

Keywords: Cryptococcal antigens, cryptococcosis, Colombia, immunoglobulins, controls

Introduction

Cryptococcosis is a life-threatening infectious disease of global distribution caused by the encapsulated yeasts *Cryptococcus neoformans* and *Cryptococcus gattii* species complexes, which affect mainly immunocompromised patients and, in less extent, immunocompetent people or without evident predisposing factors.[1] Very closely after tuberculosis, cryptococcosis is the second leading cause of death in people living with HIV.[2] In Colombia, its annual incidence in the general population is 2.3 cases per million inhabitants, while in people living with HIV the incidence increases to 1.1 cases per thousand patients.[3] In addition, an average annual incidence of 0.017 cases per thousand children under 16 years have been reported in Colombia.[4] Remarkably, the state of Norte de Santander has the highest incidence of cryptococcosis in the general population in the country (0.56 cases per 100 000 people).[3]

Cryptococcal infection is initiated by the inhalation of desiccated yeasts or basidiospores, which are ubiquitous in the environment, mainly in soil, avian excreta, several species of trees, and decaying wood.[5,6] The infection presents initially as pneumonia, but after dissemination to the central nervous system, it presents as meningoencephalitis, the most frequent form, which can be fatal if it is left untreated.[7] In middle-income countries, including Colombia, mortality from meningeal cryptococcosis is estimated to range between 34 to 46% of the cases receiving amphotericin B plus fluconazole as antifungal therapy, and up to 70% of the cases that do not receive appropriate treatment.[2,8]

As a consensus regarding the adoption of the new nomenclature for *C. neoformans* and *C. gattii* has not yet been reached,[9,10] to avoid confusion, in this study the cryptococcal species are referred to either as *C. neoformans* or as *C. gattii*. Even though > 80% of cases of cryptococcosis, in

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patients with an identified underlying immunocompromised condition, are caused by *C. neoformans*, *C. gattii* causes most cases in individuals with an apparently normal immune system. As such, while *C. neoformans* has long been considered an opportunistic pathogen, *C. gattii* has been commonly designated as a primary pathogen.[1] Increasing evidence, however, has shown that a significant percentage of the patients affected by *C. gattii* have anti-granulocyte-macrophage colony-stimulating factor autoantibodies, apart from other possible subtle alterations in their immunity that facilitate infection by this species, which makes it, therefore, an opportunistic pathogen.[11–13] Nevertheless, clinical data suggest that infections caused by *C. gattii* are significantly more severe than those caused by *C. neoformans*, since *C. gattii* induces massive inflammation and formation of cryptococcomas, which are associated with worse neurological sequela and require additional clinical follow-up as well as longer periods and higher doses of antifungal treatment.[14] In addition, as *C. gattii* cryptococcosis prevails in patients who are not evidently immunocompromised, these patients will generally have increased mortality risk, which is largely driven by delayed diagnosis from the non-classical clinical presentation and lack of screening tools.[15] The geographical distribution of these two species also differs. While *C. neoformans* has a worldwide distribution, *C. gattii* predominates in tropical and subtropical areas, with an on-going extension of its ecological niche to temperate zones, especially to Vancouver Island in Canada and the Pacific Northwest of the United States, where it has caused outbreaks of infection.[1,16] In Colombia, about 4% of cryptococcosis cases have been reported to be caused by *C. gattii*, with > 90% of cases being from patients with no defined risk factors.[17] Although *C. neoformans* infection is known to be acquired at an early age, cryptococcosis in children is very rare. In the literature, < 500 cases of pediatric cryptococcosis have been reported.[4,18]

It is known that cryptococcal infection elicits a humoral response with the generation of both protective and nonprotective antibodies,[19,20] although the role of these antibodies has not been fully understood and there are no clinical and serological studies that associate the presence and class of antibodies with the persistence of the infection, relapse, reactivation, and re-infection. Several serological studies, however, have determined the reactivity and established the prevalence of antibodies against the polysaccharide glucuronoxylomannan (GXM) the major component of the cryptococcal capsule, in different patients and control groups.[21,22] In contrast, fewer studies have explored the antibodies produced against specific non-capsular protein antigens from both major cryptococcal species.

Immunoreactive proteins of *C. neoformans* and *C. gattii* that induce protective immune responses in mice have been described.[23–25] In addition, disease-associated protein antigens that have a role in the pathogenesis of cryptococcosis in rodents and koalas, have been identified by immunoproteomics.[19,26,27] In humans, *C. neoformans* and *C. gattii* protein antigens that react with immunoglobulin (Ig)G have also been recognized in diverse populations, including adults with cryptococcosis, with and without HIV infection, as well as in healthy children and adults.[19,20,28,29] Apart from IgG, *C. neoformans* specific proteins that react with IgM and IgA have been identified in cryptococcosis patients and healthy people.[30–32] More recently, by an immunoproteomic approach and subsequent recombinant expression, disease-associated *C. neoformans* proteins reactive with IgG antibodies were identified in serum pools from adult patients with and without cryptococcosis from Colombia.[33] However, until now, the antibody levels to specific protein antigens from both *C. neoformans* and *C. gattii*, in a large number of sera, including sera from children with cryptococcosis, have not been reported.

This study aimed, therefore, to recognize the reactivity of proteins from the two main agents of cryptococcosis that specifically react with IgG, IgM and IgA antibodies present in sera from children and adults, with and without diagnosis of cryptococcosis, from Colombia. This investigation allowed as well to determine that patients with cryptococcosis and people without history of this mycosis present surprisingly similar *C. neoformans*- and *C. gattii*-specific antibodies, which vary with the age, presuming differential environmental exposure. The findings of this study are the basis to identify IgA- and IgM-immunoreactive proteins that could play a role in cryptococcosis defense.

Materials and methods

Sera collection

Sera samples obtained from 109 patients with diagnosis of cryptococcosis and from 119 people with no apparent cryptococcal infection or any other infectious disease (controls) were included in the study. Cryptococcosis was diagnosed by blood culture and/or by detecting cryptococcal antigen (CrAg) in sera, based on the CrAg latex agglutination system (CALAS®) (Meridian Bioscience). *Cryptococcus* species were identified by phenotypic methods, including urease activity and glycine assimilation on L-canavanine-glycine-bromothymol blue medium, as described previously.[34] Sera from patients with cryptococcosis, belonging to the sera collection of the Microbiology Group of the National Institute of Health in Bogota, Colombia have been collected between 1999 and 2019, mostly from Bogota (56.9%) and Norte de Santander (33.9%), but also from Huila (4.6%), Magdalena (1.8%), Bolivar, Boyaca and Tolima (0.9% each). From the cases of cryptococcal infection, 81 (74.3%) were caused by *C. neoformans* and 28 (25.7%) by *C. gattii*. The characteristics of these patients, registered as part of the National Surveillance Program for *Cryptococcus* and cryptococcosis in Colombia, led by the National Institute of Health, are summarized in Tables 1 and 2. Infection with HIV was reported in 55 patients (50.5%), predominantly affected by *C. neoformans* infection (98.2%). Most cases were from adult men (63.3%). In the studied population, adults were 5.34 more likely to be diagnosed with cryptococcosis than children (95% confidence interval [CI] 2.52-11.34, $P < .001$), and men were 9.92 times more likely to be diagnosed with cryptococcosis than women (95% CI 5.23-18.81, $P < .001$). Because the number of cases of cryptococcosis in children is much lower compared to the number of cases in adults,[4] only 10 available sera samples from children, all affected by *C. neoformans*, were included. From this group of patients, 5 (50%) were HIV positive. Underlying conditions, other than HIV, such as transplant, malignancy, diabetes, tuberculosis, among others, were not reported among any of the patients with cryptococcosis.

Serum from adults and children without cryptococcosis were recovered in 2019 from voluntary participants in Bogota (68.9%), Norte de Santander (27.7%), and Boyaca

Table 1. Distribution of the number of patients with cryptococcosis, according to HIV status, sex, and etiological agent.

		HIV status			
		HIV+	HIV-	No data	Total
Sex	Male	49	29	14	92
	Female	6	9	2	17
Etiological agent	*Cryptococcus neoformans*	54	15	12	81
	Cryptococcus gattii	1	23	4	28
	Total	55	38	16	109

Table 2. Distribution of the number of patients with cryptococcosis and controls, according to age and sex.

	***n* of patients per age (*n* of patients per sex)**			
Population	**Adults (M/F)**	**Children (M/F)**	**No data (M/F)**	**Total (M/F)**
Cryptococcosis	79 (69/10)	10 (8/2)	20 (15/5)	109 (92/17)
Controls	71 (25/46)	48 (17/31)	0	119 (42/77)
Total	150 (94/56)	58 (25/33)	20 (15/5)	228 (134/94)

M: male; F: female.

(3.4%). The first two states were selected considering that they represented the higher number of cryptococcosis cases included in the study and that, historically, Norte de Santander has a notable high rate of cryptococcosis in the country.[3] Sera from control people were stored in the MICROS Group laboratory in Universidad del Rosario, Bogota, Colombia. Age and sex of individuals without cryptococcosis are detailed in Table 2.

For the purposes of this study, an adult was defined as anyone whose age was older than 19 years and a child as anyone between 0 and 19 years, following the definitions of the guidelines on the diagnosis, prevention, and management of cryptococcosis in HIV-infected people.[35]

Protein extraction from cryptococcal cells

The reference strains of *C. neoformans*, H99, and *C. gattii*, H0058-I-2029, which represent the most common genotypes of these species causing disease in Colombia, VNI and VGII, respectively,[3] were used for the protein extraction. Total cryptococcal proteins, which include cell wall-associated proteins together with cytoplasmic proteins, were extracted separately from both strains as previously reported,[27,33] with some modifications. Briefly, each strain was recovered from 10% glycerol stored at −80°C by plating it on Sabouraud dextrose agar for 48 h at 27°C. From each culture, a single colony was transferred in Sabouraud dextrose broth and incubated with agitation at 80 rpm and 30 °C for 48 h. Afterward, cells were harvested by centrifugation and washed twice with 250 mM sucrose. About 5 g of centrifuged cells were then resuspended by pipetting in 5 ml of 2X lysis buffer (10 mM Tris/HCl pH 7.5 supplemented with 5 mM EDTA and 1X protease inhibitor [Roche]) and 5 ml of a solution containing 8% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and 100 mM dithiothreitol (DTT). This suspension was placed in a mortar, the cells were frozen with liquid nitrogen and homogenized twice consecutively, by maceration with a pestle. To concentrate whole-cell protein samples and to remove contaminants, including salts, detergents, and carbohydrates, proteins were recovered by centrifugation, after trichloroacetic acid (TCA) precipitation and the concentration was measured with the NanoDrop™One/One (ThermoFisher Scientific) using the Bradford method (ThermoFisher Scientific). Proteins samples were kept frozen at −20 °C until further immunoglobulin isotyping.

Total immunoglobulins quantification

Total levels of IgG, IgA, and IgM from all sera were quantified by ELISA, as previously described.[27,33] Briefly, 96 well round bottom plates were coated with 50 µl of goat anti-human-IgG, -IgA, or -IgM, respectively (SouthernBiotech), diluted 1:1000 in carbonate buffer and incubated overnight at 4 °C. The plates were washed once with phosphate buffered saline (PBS) and 0.05% Tween-20 (PBST), blocked with blocking buffer (1X PBS, 0.5% bovine serum albumin [BSA] and 0.1% gelatin) for 1 h at room temperature and washed twice with PBST. Human IgG (0.5 µg/ml), IgA (2 µg/ml), and IgM (2 µg/ml) (SouthernBiotech) were used as standards, respectively. Sera were suspended in blocking buffer containing 0.05% Tween-20, at a dilution of 1:100 000 for IgG and 1:2000 for IgA and IgM. Each diluted serum was placed in triplicate. The plates were incubated with the standards and serum samples for 1.5 h at room temperature and washed three times with PBST. Detection was done with goat anti-human-IgG, -IgA, and -IgM, respectively, labeled with horseradish peroxidase (HRP) (SouthernBiotech) and diluted 1:4000. After 2 h incubation, the plates were washed four times with PBST and developed with 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma-Aldrich). Immediately after the wells with the higher concentration of the standard antibody reached an OD of 1.3 at 650 nm, developing of the plates was stopped by adding 0.5 M H_2SO_4 (Sigma-Aldrich). A final reading of the plates was done at 450 nm and the concentration of each immunoglobulin isotype per serum was calculated using the software from the accuSkan FC microplate photometer (ThermoFisher Scientific).

Cryptococcal-specific immunoglobulins determination

Specific IgG, IgA, and IgM against *C. neoformans* and *C. gattii* total proteins were determined, separately, for all serum samples as previously described,[27,33] with various modifications. Per well, ELISA plates were coated overnight with 0.5 µg of total proteins of *C. neoformans* or *C. gattii*, respectively, and incubated overnight at 4 °C. The plates were washed once with PBST and blocking was done with 5% skim milk dissolved in PBS (SM). Sera were suspended in SM containing 0.05% Tween-20 (SMT), at a dilution of 1:100 for IgG and 1:25 for IgA and IgM. Each diluted serum was placed in duplicate in the plate, due to the restricted volume of sera. Detection was done with HRP-goat-anti-human-IgG, -IgA, or IgM, respectively (SouthernBiotech), diluted 1:4000. Development of the plates was done with TMB for 15 min at room temperature in the dark and stopped with 0.5 M H_2SO_4 prior to OD determination at 450 nm. The detection limit to determine specific IgG, IgA, and IgM against cryptococcal proteins was an OD < 4.0. For all ELISA experiments, wells incubated without sera samples but with all other reagents were used as blanks. Titration of sera was not done due to the small volume of most samples. Cryptococcal-specific antibody levels were determined following standardized protocols for the verification of cryptococcal specificity, including i) coating the plates with proteins from *Candida albicans*, ii) using sera from patients with other mycosis such as histoplasmosis and paracoccidioidomycosis, and iii) using IgG isolated from rabbits immunized with *C. neoformans* (Rabbit anti-Cn IgG), as previously reported.[27,33]

Statistical analysis

Mean and standard deviation were calculated for continuous variables, while relative frequencies were calculated to describe categorical variables. Depending on the distribution of the data, Mann-Whitney nonparametric test for unpaired samples, 2-tailed Student test (t-Test) for unpaired samples, or 2-tailed Fisher's exact test for categorical data were performed to determine statistical differences in the total and specific levels of immunoglobulins between groups, according with the ELISA results. A Wilcoxon signed-rank test was used to compare two related samples. The Pearson correlation coefficient (ρ) was used to assess the relationship between continuous variables. Correlation was judged very strong from 1 to 0.8, strong from 0.8 to 0.5, fair from 0.5 to 0.2, and poor from 0.2 to 0. Normality of data was assessed with the Shapiro-Wilk test. Alpha risk was set to 5% ($\alpha = 0.05$). Simple linear regression was used to describe the relationship between dependent and independent variables. A multivariate linear regression was performed to assess the relation between continuous and explanatory variables. Statistical analysis was performed with the online application EasyMedStat (version 3.9; www.easymedstat.com) and Jamovi 1.6.23. GraphPad Prism v 7.05 software was used to graph the results. Statistical significance is presented as follows: * $P \leq .05$, ** $P \leq .01$, *** $P \leq .001$, and **** $P \leq .0001$.

Ethics

Patients' identification was anonymized for the purpose of this study, which was carried out in accordance with the Technical Committee of Research (CTIN) and the Ethical Committee for Research (CEIN) of the National Institute of Health, Bogota, Colombia, protocol CEMIN N° 37-2017, as well as with the Research Ethics Committee of Universidad del Rosario (CEI - UR), protocol DVO005 931-CV1073. A waiver of the informed consent for the use of sera from patients with cryptococcosis was included in the document CEMIN N° 37-2017. Serum from people without cryptococcosis were recovered after signing informed consent.

Results

Serum IgG levels increase during cryptococcosis while serum IgA and IgM levels are higher in control individuals

Production of total IgG was determined to be higher in patients with cryptococcosis than in controls, independently on HIV status and age. Among patients with cryptococcal infection, serum IgG levels were higher in HIV-positive (HIV+) compared with HIV-negative (HIV−) (16 381.48 vs. 11 207.23 µg/ml) ($P = .0015$) (Fig. 1a), as well as in adults compared with children (14 272.04 vs. 12 579.98 µg/ml), although this difference was statistically non-significant ($P = .71$) and there was no correlation between IgG levels and age ($P = -.060$, $P = .58$).

Contrarily, serum IgA and IgM levels were generally higher in controls than in all patients with cryptococcosis, independent of age. However, while the IgA levels were lower during cryptococcal infection, independently on the HIV status (Fig. 1b), IgM levels were significantly lower in HIV− patients (497.00 µg/ml) ($P < .0001$) but just slightly lower in HIV + patients, compared with control individuals (911.47 *vs*. 1014.72 µg/ml) ($p = 0.12$) (Fig. 1c). Adults with cryptococcosis presented statistically higher levels of both IgA and IgM than children with cryptococcosis (541.68 vs. 199.35 µg/ml for IgA and 776.13 vs. 697.88 µg/ml for IgM) ($P = .0073$ and $P = .03$, respectively), although there was no correlation between the total level of these antibodies and age ($P = .141$, $P = .19$ for IgM, and $\rho = -0.043$, $P = .7$ for IgA).

Patients infected with *C. neoformans* produced significantly more IgG (14 954.94 vs. 11 012.94 µg/ml) ($P = .01$) and IgM (860.16 vs. 531.35 µg/ml) ($P = .009$) than those infected with *C. gattii*, as well as more IgA (500.07 vs. 455.32 µg/ml), although the difference was statistically non-significant ($P = .33$). Total levels of IgG, IgA and IgM did not differ among cryptococcosis patients nor among controls coming from different geographical regions (Bogota vs. Norte de Santander) ($P > .05$). Total antibody levels did not differ either among men or women with or without cryptococcosis ($P > .05$).

Cryptococcal-specific IgG, IgA, and IgM are produced during cryptococcosis as well as in control individuals, differing between adults and children

After quantification of total immunoglobulin concentrations, specific IgG, IgA, and IgM that were reactive with total proteins of *C. neoformans* (*Cn*) and *C. gattii* (*Cg*), were determined separately. Notably, the reactivity of IgG and IgM was significantly higher with proteins from *C. neoformans* than from *C. gattii*, as established by the determined OD, not only in sera from cryptococcosis patients but also in sera from control individuals (Fig. 2a). Although in patients with cryptococcosis the reactivity of IgA with *C. neoformans* proteins

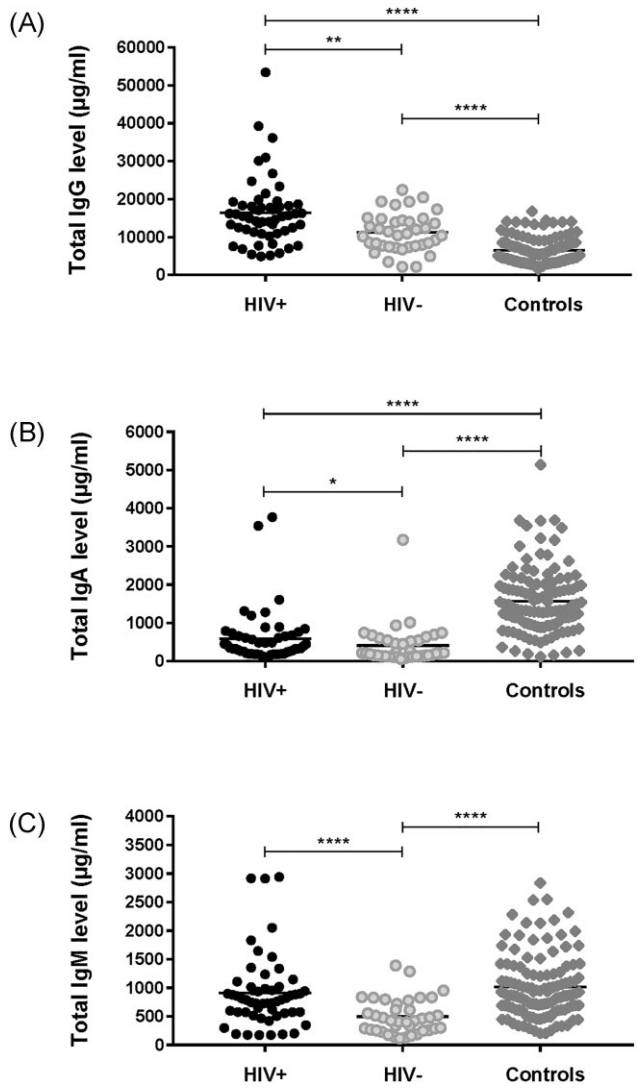

Figure 1. Total levels of serum IgG, IgA and IgM in cryptococcosis patients and controls. Patients with cryptococcosis, both with HIV infection (HIV+) and without HIV (HIV−), presented higher levels of total IgG compared to controls (A). Opposite, people without cryptococcosis produced higher IgA (B) and IgM (C) levels compared to cryptococcosis patients. Each spot represents a serum sample of an individual person. The median value is shown by a horizontal line. Statistical significance, using the Mann-Whitney nonparametric test for unpaired samples, is shown as * $P \leq .05$, ** $P \leq .01$, and **** $P \leq .0001$.

was also significantly higher than the reactivity of this antibody with *C. gattii* proteins, in people without cryptococcosis IgA reacted significantly more with *C. gattii* than with *C. neoformans* proteins (Fig. 2b). In both studied groups, cryptococcosis patients and controls, a strong positive linear correlation was found between *Cn*- and *Cg*-specific IgG ($\rho = 0.6$ and $P < .0001$), *Cn*- and *Cg*-specific IgA ($\rho = 0.94$ and $P < .0001$), as well as *Cn*- and *Cg*-specific IgM ($\rho = 0.92$ and $P < .0001$).

ODs for specific IgG against both *C. neoformans* and *C. gattii* proteins were higher in adults than in children, with and without cryptococcosis (Figs. 3a and b), although the differences were not always statistically significant. In addition, in both studied groups, a positive linear correlation was found between specific IgG and age ($\rho \leq 0.22$ and $P \leq .05$). Similarly, ODs for specific IgA (Figs. 3c and d) and IgM (Figs. 3e and f) against both *C. neoformans* and *C. gattii* proteins tended to be higher in adults, compared with children, although none of these differences had statistical significance. No or negligible correlation was found either between specific IgA or specific IgM and age ($\rho \leq 0.1$ and $P > .05$) (Data not shown). Independently of the cryptococcal species, the reactivity of specific IgG, IgA, and IgM antibodies did not differ among adults with and without cryptococcosis, nor among children of both groups ($P > .05$). Only a slightly significant difference was found between *Cn*-specific IgG in children (Fig. 3a) and *Cn*-specific IgA in adults (Fig. 3c).

In patients with cryptococcosis, specific IgG, IgA and IgM against *C. neoformans* and *C. gattii* proteins did not differ with the HIV status and sex, nor with the species causing the infection or the geographical region ($P > .05$).

In all sera from adults and children with or without cryptococcosis, a positive linear correlation was found between total and specific levels of each antibody IgG, IgA, and IgM, independently of the cryptococcal species from which the proteins were extracted. However, statistical significance of the correlation differed among antibodies (Fig. 4).

Discussion

The role of humoral immunity in fighting against cryptococcosis remains uncertain. However, several antibodies, predominantly IgG, which are reactive with GXM, melanin as well as with diverse cryptococcal proteins, have been long identified, not only in patients with cryptococcosis but also in hosts without clinical manifestations or without a history of previous cryptococcal infection.[21,22,36] Our study supports these findings, as it shows that not only serum IgG, but also IgA and IgM antibodies react with protein antigens of both *C. neoformans* and *C. gattii*, and that these antibodies are present not only in sera from adults with and without cryptococcal infection but also in sera from children with cryptococcosis, whose cases are very uncommon to encounter, as well as in sera from apparently healthy children.

Similar to other studies, in the cohort from Colombia, cryptococcosis patients with HIV presented significantly higher total IgG, IgA, and IgM levels than those without HIV infection, which could be related with the defects in cell-mediated immunity and hypergammaglobulinemia notable in HIV-infected people.[37–39] Notably, patients with cryptococcosis caused by *C. neoformans* produced much higher levels of all antibodies, compared with patients infected by *C. gattii*. This finding correlates with the number of HIV-infected people that are affected by each species, which, in our study, accounted for 66.7% of the *C. neoformans* cases and only 3.6% of the *C. gattii* cases. Globally, cases of *C. gattii* infection affecting patients with HIV are reported rarely, as it occurs in Colombia.[8,14,40] In addition, when analyzing patients with cryptococcosis but without HIV, the levels of IgG, IgA, and IgM did not differ among patients affected with one or the other etiological agent.

While total IgG levels were much lower in our control group, total IgA and IgM levels seem to decrease significantly during cryptococcosis, independently on the HIV status, which contrasts with other reports. The relationship between serum immunoglobulin levels and HIV could however differ among studies, not only because of the methodology used to measure antibody concentrations, but also because the immune response depends on the clinical stage of the HIV

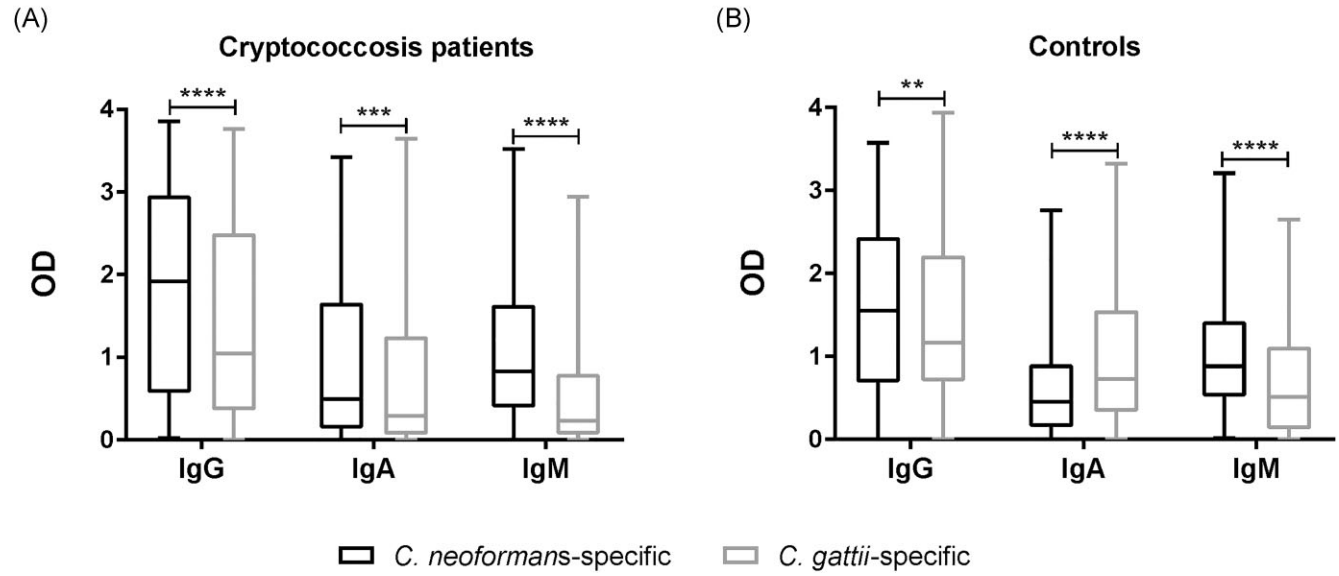

Figure 2. Reactivity of IgG, IgA, and IgM with *Cryptococcus neoformans*- and *Cryptococcus gattii*-proteins, in sera from patients with cryptococcosis and controls. The reactivity of IgG, IgA, and IgM with *C. neoformans* (*Cn*) and *C. gattii* (*Cg*) proteins were measured and expressed as optical densities. In both studied groups, cryptococcosis patients (A) and controls (B), *Cn*-specific IgG and *Cn*-specific IgM were more reactive than *Cg*-specific IgG and *Cg*-specific IgM, respectively. While serum IgA from patients with cryptococcosis were also more reactive with *C. neoformans* proteins, serum IgA from controls were more reactive with *C. gattii* proteins. The median and range values are shown. Boxes extend from the first to the third quartile. Statistical significance, using the Wilcoxon signed-rank test, is shown as ** $P \leq .01$, *** $P \leq .001$ and **** $P \leq .0001$.

infection of patients, which is unknown in our study, as well as on the effect of cryptococcosis itself and the etiological agent causing the mycosis.[32,41–43]

Reactivity of IgG, IgA, and IgM antibodies against specific cryptococcal proteins, which directly correlated with total levels of IgG, IgA, and IgM, respectively, tended to be higher in patients with cryptococcosis compared to people without this fungal infection. Although these differences were not always statistically significant, there were still some differences and trends, that perhaps can be clinically relevant.[44,45] Higher levels of specific antibodies might be elicited by cryptococcal proteins during infection, which has been reported by other investigators, not only in murine but also in human cryptococcosis.[19,23,24,32,46] However, preexisting IgG, IgA, and IgM that are reactive with GXM, proteins and mannoproteins from *C. neoformans* have been reported in children and adults without a history of cryptococcosis, which has been suggested to be due to the continuous and recurring environmental exposure to the fungi that could trigger basal production of anti-cryptococcal antibodies.[19,20,28,30,32,33] In the long run, immunoproteomic analysis of the IgG, IgA, and IgM responses to specific cryptococcal proteins could help identify immunodominant antigens that are protective, non-protective, or detrimental during cryptococcosis. It is known that, depending on isotype, specificity, and even host factors, certain antibodies can alter the course of a fungal infection to the benefit or damage of the host.[42,47]

When comparing the reactivity of IgG, IgA, and IgM with protein antigens from each of the main etiological agents of cryptococcosis, we found that all serum immunoglobulins react significantly more with *C. neoformans* proteins than with proteins of its sibling species, *C. gattii*. Presuming environmental exposure, these findings agree with the fact that, not only in Colombia, but also globally, *C. neoformans* prevail in ecological niches over *C. gattii*.[1,6,8] In Australia and Papua New Guinea, where *C. gattii* infections are much more common than those by *C. neoformans*,[14,48] some sero-epidemiological differences, depending on the etiological agent causing the infection, have been reported. Patients with *C. gattii* cryptococcosis from Australia presented higher levels and a higher prevalence of IgG and IgA against GXM than those with *C. neoformans* infection, possibly because *C. gattii* is more immunogenic and the infection takes longer to be diagnosed.[22] However, opposite to our study, the study in Australia did not determine the prevalence of antibodies against *C. gattii* specific proteins but only against polysaccharide antigens of *C. neoformans*, which leads to a differential interpretation of the results. In Papua New Guinea, adults with *C. gattii* meningitis were reported to have higher IgG levels against non-capsular protein antigens of the same species compared to children and healthy patients,[49] which is comparable with a noticeable finding in our study. In the Colombian cohort, serum immunoglobulins, especially IgG, from adults with and without cryptococcosis, tended to be more reactive with cryptococcal proteins than immunoglobulins from children with and without cryptococcosis, respectively, with an observation that antibody reactivity increases with age. Taken together, our findings strongly support the notion that, since childhood, people experience chronic exposure to spores or small desiccated yeast cells of both cryptococcal species, mainly *C. neoformans*. The finding of specific IgG, IgA, and IgM against cryptococcal proteins in sera from children without crytococcosis, as young as 1-year-old, allowed in addition to estimate that in Colombia, people are exposed to the fungus at a very young age. Similar to our findings, studies from the Bronx in New York reported that children between 1.1 to 2 years old were the younger group whose sera demonstrated reactivity against *C. neoformans* proteins.[20] Considering that the number of sera from children analyzed in our study is fairly low, which associates with the fact that pediatric

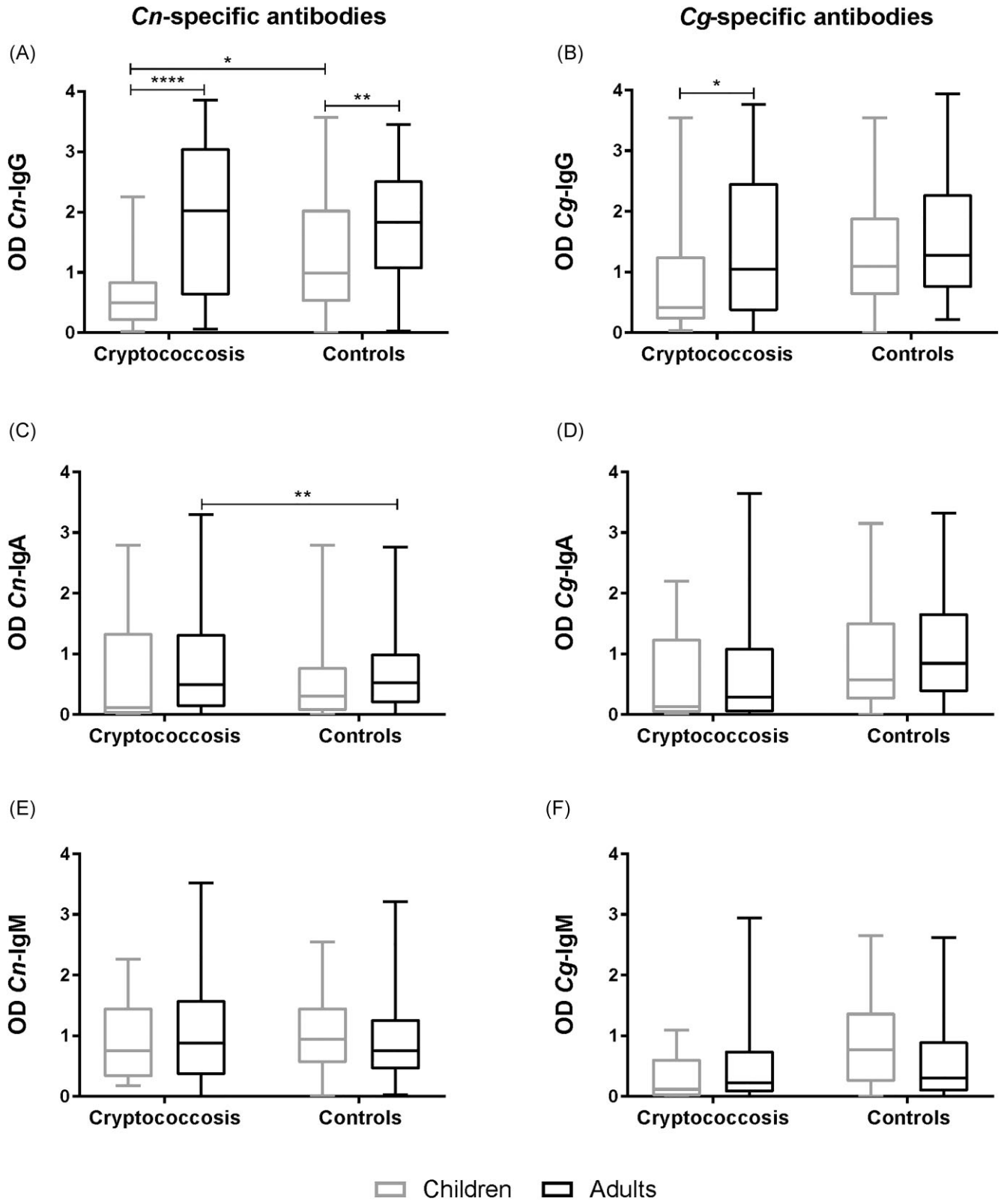

Figure 3. Reactivity of IgG, IgA and IgM with *Cryptococcus neoformans*- and *Cryptococcus gattii*-proteins in children and adults with and without cryptococcosis. The reactivity of IgG, IgA and IgM with *C. neoformans* (*Cn*) and *C. gattii* (*Cg*) proteins were measured and expressed as optical densities. Generally, *C. neoformans* and *C. gattii* proteins were less reactive with serum IgG (A and B), IgA (C and D) and IgM (E and F) from children, with and without cryptococcosis, that with immunoglobulins from adults. Independently of age and etiological agent, sera from people without cryptococcosis tend to be as reactive with IgG, IgA and IgM as sera from patients with cryptococcosis. The median and range values are shown. Boxes extend from the first to the third quartile. Statistical significance, using the Mann-Whitney nonparametric test for unpaired samples, is shown as * $P \leq .05$, ** $P \leq .01$, and **** $P \leq .0001$.

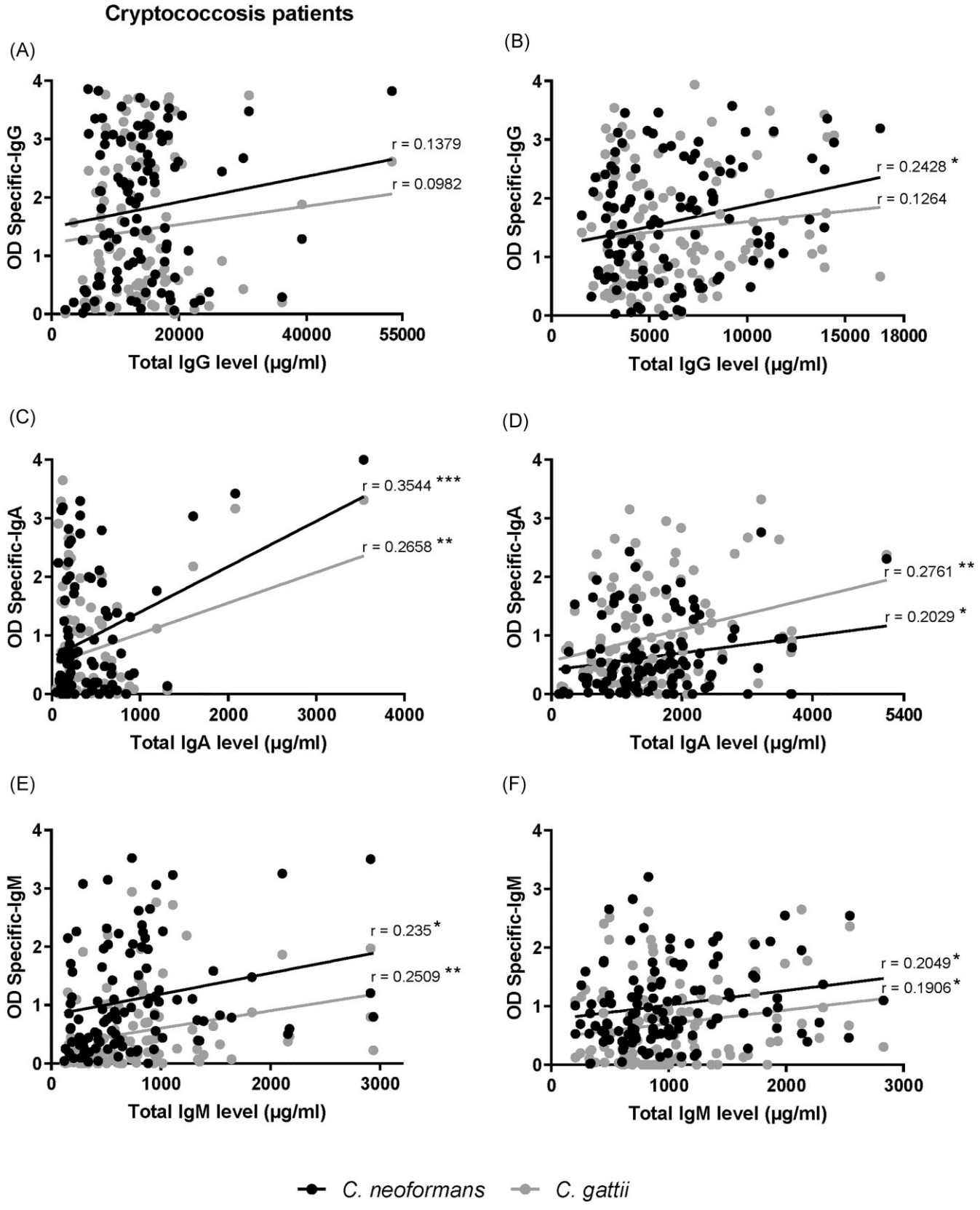

Figure 4. Correlation between total and specific IgG, IgA, and IgM in patients with cryptococcosis and controls. Total serum levels of IgG (A and B), IgA (C and D) and IgM (E and F) positively correlated with the reactivity of IgG, IgA and IgM with *C. neoformans* (black dots) and *C. gattii* (grey dots) proteins, respectively, although the correlation was not always statically significant. Each spot represents a serum sample of an individual person. The correlation, denoted by r and represented by a line, shows the amount of association between total and specific immunoglobulins. As the correlation is positive, r is between 0 and 1. Statistical significance, using the Pearson product-moment correlation coefficient, is shown as * $P \leq .05$, ** $P \leq .01$, and *** $P \leq .001$.

cryptococcosis is relatively rare, further studies including a higher number of serum samples from this age group are recommended, in order to confirm our results and to draw more meaningful conclusions. Lastly, although the prevalence of asymptomatic *C. neoformans* infection in children has been reported to vary geographically, explained possibly by an association with differences in environmental exposure to the yeast,[28] in our study, no serological differences between patients from Bogota and Norte de Santander were found, in spite of the significant difference between the incidence of cryptococcosis in these two regions,[3] which then might be due to other factors such as a genetic predisposition or immune dysfunction of the hosts or even environmental differences between these two cities.

The general observations from our study, regarding the differential IgG, IgA, and IgM responses to protein antigens, from both *C. neoformans* and *C. gattii* in patients with cryptococcosis and healthy people in different age groups might have important implications for the prevention and therapy of cryptococcosis. For instance, further proteomic studies can be done to identify reactive proteins that are disease-specific, species-specific and/or age-specific, and that can be assessed for the development of vaccines strategies not only for prevention of cryptococcosis but also aiming to control reactivation, as environmental exposure to the yeasts occurs since childhood. As it has been suggested that B cell response and antibodies are required in the normal clearance of cryptococcal cells,[50] it is possible that the specific IgG, IgA, and IgM responses to protein antigens may be of some significance, similarly to the production of antibodies against the major capsular polysaccharide GXM, which has been correlated with improved prognosis in both *C. neoformans* and *C. gattii* infections.[51,52] Differential antibody reactivity with cryptococcal proteins could lead to a reappraisal of the study of humoral immunity and its role for host defense against cryptococcosis.

Potential limitations of this study must be considered, including its retrospective design as well as the limited number and volume of samples analyzed, which together with missing data from some patients may prevent to identify significant relationships in the data. In addition, for further studies, other latent or unmeasured variables that may affect the antibody levels of patients must be taken into consideration, such as the time between cryptococcosis diagnosis and blood collection as well as if the patients were receiving antifungal therapy or antiretroviral therapy, in those with HIV, at the time of blood collection.

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Declaration of interest

The authors declare no conflict of interest.

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