



Original Article

Analysis of asthma patients for cryptococcal seroreactivity in an urban German area

Andreas Grahnert¹, Uwe Müller¹, Heiner von Buttlar^{1,†}, Regina Treudler^{2,3} and Gottfried Alber^{1,3,*}

¹Institute of Immunology/Molecular Pathogenesis, Center for Biotechnology and Biomedicine, College of Veterinary Medicine, University of Leipzig, Leipzig, Germany, ²Department of Dermatology, Venerology & Allergology, University of Leipzig, Leipzig, Germany and ³LIFE–Leipzig Research Center for Civilization Diseases, Universität Leipzig, Germany

*To whom correspondence should be addressed. Gottfried Alber, Institute of Immunology, College of Veterinary Medicine, University of Leipzig, An den Tierkliniken 11, D - 04103 Leipzig, Germany. Tel: +49-341-9738328; Fax: +49-341-9738147; E-mail: alber@rz.uni-leipzig.de

†Current address: Bundeswehr Institute of Microbiology, Munich, Germany

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Abstract

Cryptococcus neoformans is an opportunistic fungal pathogen that causes lung inflammation and meningoencephalitis in immunocompromised patients but is also able to asymptomatically infect immunocompetent individuals. *C. neoformans* is found ubiquitously especially in urban areas where it is spread by pigeons, and fungal exposure may predispose for asthma development already at an early age, as soon as confronted with pigeon droppings. In the study presented here, we investigated the presence of specific immunoglobulin G (IgG) against *C. neoformans* in sera from patients suffering from asthma in comparison to a healthy control cohort, accrued from the Leipzig Research Centre for Civilization Diseases (LIFE). For serological analysis we developed a flow cytometry (FACS) based assay specific for an acapsular strain of *C. neoformans* to comprehensively analyze different cryptococcal serotypes. Compared with the non-asthmatic cohort, asthmatics exhibited, as expected, an elevated level of total serum immunoglobulin E (IgE), whereas the IgG seroreactivity against *C. neoformans* was not significantly different among the two groups ($P = .118$). Nevertheless, there was a trend toward increased *Cryptococcus*-specific IgG antibodies in the serum of asthmatics. Additionally, in male asthmatics an increased IgG-mediated seroreactivity compared to female asthmatics was found. This points to a higher prevalence of subclinical *C. neoformans* infection in male asthmatics and may support the hypothesis of *C. neoformans* as a risk factor for the development of asthma in urban areas.

Key words: seroreactivity, *Cryptococcus neoformans*, asthma, fungal allergy.

Introduction

Asthma is a frequent and severe inflammatory respiratory disease especially afflicting people in urban environments. As estimated by the Global Initiative for Asthma (GINA) about 300 million suffered from asthma in 2004 [1]. In developed countries, asthma has more than doubled in prevalence and severity during the past 60 years [2]. For example, 24.6 million adults and children (8.2% of population) in the United States are afflicted by asthma [3]. It has been shown that fungal infections (e.g. *Aspergillus fumigatus* and dermatophytes) can exacerbate asthma [4,5]. Disease exacerbation by *A. fumigatus* is recognized as allergic bronchopulmonary aspergillosis (ABPA). This fungus causes bronchial colonization of approximately 0.7% to 3.5% of asthmatic patients [6].

Cryptococcus neoformans (*C. neoformans*), an opportunistic, facultative intracellular encapsulated basidiomycete, occurs ubiquitously in the environment and can be isolated from bird excreta and the surrounding air [7]. Infection with *C. neoformans* is believed to occur by inhalation of aerosolized particles, that is, spores or desiccated cells [8]. The fungus can cause life-threatening meningoencephalitis in immunocompromised individuals such as immunosuppressed organ transplant recipients or HIV-infected patients [9–11]. On the other hand, North American serological studies suggest that subclinical pulmonary infection with *C. neoformans* is very common among immunocompetent children especially in urban areas such as New York City [12–14]. Persistent pulmonary infection in immunocompetent individuals remains clinically unrecognized or may be misinterpreted as viral infection [15–17].

Experimental studies in mice from our own and other groups demonstrate allergic pulmonary inflammation upon intranasal infection with *C. neoformans* [18,19]. It was shown that subclinical infection of rats with *C. neoformans* enhances experimentally induced asthma by an elevated response to the allergen ovalbumin (OVA) in *C. neoformans*-infected rats in contrast to uninfected animals. Infected and OVA-treated rats show higher numbers of lung eosinophils and higher IgE titers concomitant with increased levels of mucus producing goblet cells than OVA-treated control animals. Under antifungal treatment an anti-allergic effect can be observed [20]. In mice, *C. neoformans* can cause allergic bronchopulmonary mycosis characterized by production of type 2 immune response (Th2) cytokines, elevated levels of serum IgE, recruitment of eosinophils, and alternative activation of macrophages [21–24]. Immunocompetent intranasally infected wild-type mice treated with methacholine displayed significantly elevated bronchoconstriction (bronchohyperreactivity) as compared with naive mice or interleukin 4 (IL-4)- or IL-4 receptor-deficient infected mice [18].

Given the epidemiological association between pigeon guano (urban areas with high density of these birds) and *C. neoformans* infection (see [12–14]), urban inhabitants have been found to be at particularly high risk for development of asthma [25,26]. The earliest anticryptococcal antibody titer was found in two year-old children when they start to have direct contact with the environment, by visiting play grounds where a direct contact to pigeon guano is possible [14]. The basis for the high prevalence of asthma in urban environments is unclear but likely to be multifactorial comprising environmental and lifestyle factors. Among various factors subclinical fungal pulmonary infection may predispose to asthma development of urban inhabitants [27]. The correlation between *C. neoformans* infection and asthma development is not unexpected, because the same kind of immune response (i.e., Th2) occurs in both cases. Many commonalities can be seen, as mentioned above, between asthma and allergic bronchopulmonary cryptococcosis. And a recent study of house dust-induced asthma could correlate a decreased diversity of *Cryptococcus* species with increased asthma risk [28].

Up to now studies in humans and correlations between asthma and *C. neoformans* infection were only known from North America. This is to our knowledge the first study to investigate a potential correlation between anticryptococcal seroreactivity and asthma for a European city (i.e., Leipzig and surrounding suburbs, Germany). Our study was supported by the Leipzig Research Centre for Civilization Diseases (LIFE) which studies the most important civilization diseases on a broad scale. This enabled us to undertake a first explorative analysis with a limited number of participants (i) to determine the prevalence of subclinical cryptococcal infections in humans in an urban German region, and (ii) to explore the potential contribution of subclinical pulmonary cryptococcal infection to the prevalence of asthma. Potentially, this initial study can be expanded to a more comprehensive epidemiological survey including individuals from rural areas.

Materials and methods

Study design

We included consecutive patients presenting in the allergy unit of the Department for Dermatology, Venerology & Allergology at the University Hospital of Leipzig with a history of asthma (asthma cohort) or who accrued by the Leipzig Research Centre for Civilization Diseases–LIFE (control cohort). Most asthmatics in this study had a mild form of asthma (see results). The procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation within the Medical

Faculty of the University of Leipzig (Germany) and/or with the Helsinki Declaration of 1975, as revised in 1983. The patients studied (both asthma and control group) were all immunocompetent with no signs indicative for any opportunistic infection. Serum GXM analysis of the samples described in Figure 3D revealed no detectable GXM levels (data not shown).

Determination of serum IgE levels and eosinophils

Blood and serum samples were routinely tested at the Institute of Laboratory Medicine, University of Leipzig, for IgE levels (CAP FEIA system, Thermo Fisher, Freiburg, Germany) and eosinophils.

Cultivation of *Cryptococcus neoformans*

The acapsular *C. neoformans* strain CAP67 originally derived from serotype D strain B3501 [29] was kindly provided by Dr. B. Fries (Albert Einstein College of Medicine, Bronx, NY), kept as a frozen stock in skim milk and grown in Sabouraud dextrose medium (2% glucose and 1% peptone; Sigma, Deisenhofen, Germany) for 72 h on a shaker at 30 °C. Cells were washed twice in sterile phosphate-buffered saline (PBS), resuspended in PBS containing 0.3% (v/v) Tween® 80 (PBS/T) (Sigma-Aldrich, Deisenhofen, Germany), and counted in a hemacytometer.

Detection of *C. neoformans*-specific IgG antibodies by flow cytometry (FACS)

In sum, 1×10^6 cells of *C. neoformans* CAP67 were incubated in the presence of 100 μ l Fixable Viability Dye eFluor® 780 (eBioscience, Frankfurt, Germany) (dilution 1:500) for 25 min. After washing with PBS/T, 100 μ l serum (dilution 1:200 in PBS/T) was added and incubated for 30 min. Additionally, as a positive serological control we used protein G purified rabbit serum IgG from a rabbit that had been immunized using heat-inactivated *C. neoformans* (serotype D, 1841 strain) cells; 0.5 μ g of both rabbit anti-*C. neoformans* IgG and the respective isotype control (Southern Biotechnology Associates, Birmingham, AL, U.S.A.) per 1×10^6 cells were incubated with the fungus as described above. To compare the antibody responses of healthy or asthmatic humans with experimental *Cryptococcus neoformans* infection of mice, we analyzed sera of naïve and infected Balb/cJ wild-type mice (70 days post infection). Balb/cJ mice were infected intranasally with *C. neoformans* strain 1841 (inoculum 500 colony forming units) and the sera were analyzed as described above. Following incubation with FITC-labeled goat anti-human IgG antibody

(Southern Biotechnology Associates), Alexa Fluor® 488-labeled goat anti-rabbit IgG (Life Technologies, Darmstadt, Germany), or FITC-labeled rat anti-mouse IgG antibody (Biolegend, Fell, Germany) cells were fixed and acquired on a LSRFortessa (BD Biosciences, Heidelberg, Germany). Exclusion of dead cells which were observed in average at about 4% and found to show a somewhat higher MFI than live cryptococci upon incubation with serum (possibly due to nonspecific antibody binding) and exclusion of doublets (to study single cells) was performed prior to determination of geometric mean fluorescence intensities (MFI). Data were analyzed using FlowJo 7.6.5 (Treestar Inc., Ashland, OR, USA) software.

Detection of *C. neoformans*-specific Ig (IgG, IgM, IgA) antibodies by ELISA

C. neoformans CAP67 cells (5×10^5 /ml) were grown in 200 ml Sabouraud dextrose medium for 72 h on a shaker at 30 °C. After harvesting and washing with PBS cells were resuspended in lysis buffer containing 10 mM Tris/HCl pH 7.5 (Sigma-Aldrich), 5 mM EDTA (Sigma-Aldrich), and complete protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). Protein extracts were obtained using the Precellys® 24 homogenizer and Precellys® Homogenization Kit with 1.4 mm ceramic and 0.1 mm glass beads (Peqlab Biotechnology GmbH, Erlangen, Germany). After protein determination using Bradford reagent and bovine serum albumin as reference (Sigma-Aldrich) homogenate was stored in aliquots at -80 °C.

For determination of *C. neoformans*-specific serum antibodies, the following ELISA was used. ELISA plates (Nunc GmbH & Co KG, Langenselbold, Germany) were coated with 1 μ g/ml *C. neoformans* homogenate in carbonate buffer (pH 9.5) or carbonate buffer alone (for control) overnight at 4 °C. To prevent unspecific binding, plates were washed and blocked with 5% (w/v) skim milk (Sigma-Aldrich) and 0.05% (v/v) Tween 20 (Karl Roth AG, Karlsruhe, Germany) in PBS for 3 h at room temperature. After washing, serum samples in serial dilutions starting from 1:20 in blocking buffer were added in duplicates for 2 h at room temperature. After washing, this step was followed by incubation with peroxidase-labeled goat-anti-human Ig (Southern Biotechnology Associates), diluted 1:30,000 in Tween® 20 containing blocking buffer, for 2 h at room temperature for detection. Plates were washed, and the TMB Microwell peroxidase system (KPL, Gaithersburg, MD, U.S.A.) was used as a substrate for the final colorimetric reaction. The reaction was discontinued after 45 min by adding 1 M H₃PO₄, and ODs were read using a Spectra-max 340 ELISA reader (Molecular Devices GmbH, Munich, Germany) at 450 nm (reference at

630 nm). ΔOD_{450} was used for analysis and calculated by subtracting the mean OD_{450} of the 1:160 dilution of serum samples incubated carbonate buffer treated wells from the mean OD_{450} of serum samples (same dilution) incubated in homogenate-coated wells.

Confirmation of anti-*C. neoformans* specificity of serum samples

In order to test whether the detected reactivity of serum samples against *C. neoformans* was not due to the presence of cross-reactive antibodies (e.g., originally directed against *Candida albicans*) we developed a FACS based test system. Briefly, 50 μ l of serum samples (dilution 1:200) were preabsorbed by incubation with 4×10^6 cells of *C. neoformans* CAP67, H99 (serotype A) or *C. albicans* (strain SC5314) for 30 min at 4 °C. After centrifugation, supernatants were transferred to 1×10^6 cells of *C. neoformans* CAP67 and incubated for additional 30 min at 4 °C. For visualization 50 μ l of a FITC-labelled goat-anti-human IgG antibody were used. For control, serum samples without preabsorption were used for CAP67 incubation (untreated control) and the MFI of untreated vs. preabsorbed samples was calculated for analysis. Four different combinations were used: (i) untreated: serum sample + 1×10^6 CAP67, (ii) CAP67 absorbed: serum sample + 4×10^6 CAP67 > CAP67 absorbed sample serum + 1×10^6 CAP67, (iii) H99 absorbed: serum sample + 4×10^6 H99 > H99 absorbed sample serum + 1×10^6 CAP67, (iv) SC5314 absorbed: serum sample + 4×10^6 SC5314 > SC5314 absorbed sample serum + 1×10^6 CAP67.

Statistical analysis

Comparison of the mean ages of participants of the two groups was done by unpaired Student's *t*-test. Fisher's exact test was used to compare proportions of gender between the two groups. IgE concentrations and frequency of eosinophils were analyzed by Mann-Whitney test. Initially, when comparing the MFI values of gender- and age-groups (depicted in Fig. 5 and data not shown, respectively) we used the Kruskal-Wallis test. We did not account for multiple testing due to the explorative character of this study. Nevertheless, depending on the passed or failed Kolmogorov-Smirnov normality test, comparisons of MFI values were done using the unpaired Student's *t*-test or Mann-Whitney test, respectively. When calculating the sample size for unpaired *t*-test with a desired power of 0.8 and the mean difference and standard deviation of the explorative experiment shown in Figures 3 and 5 (analyzing *Cryptococcus*-specific antibody levels), a sample size of approx. 500 participants is necessary to reach a statistically significant conclusion.

Here only 68 asthmatics and 66 controls were available. Therefore, this survey was used to deliver basic data to design a consecutive larger study in the future. To test for the correlation between seroreactivity determined by FACS analysis (MFI) and by ELISA (OD values) Spearman's rank correlation coefficient was determined.

All statistical analyses were done using GraphPad Prism® software (GraphPad Software, Inc., La Jolla, CA, USA) and calculation of sample size was done using GraphPad StatMate2 software.

Results

Infection with *Cryptococcus neoformans*, a common opportunistic fungal pathogen, mainly occurs by inhalation of dust particles (e.g., soil, pigeon excreta) that had been contaminated with cryptococcal spores or desiccated cells [8]. In this study we wished to determine the prevalence of subclinical cryptococcal infections in humans in an urban region (i.e., city of Leipzig and its surrounding) in the Eastern part of Germany and to explore the potential contribution of subclinical pulmonary cryptococcal infection to the high prevalence of urban asthma.

Test participants

Serum samples from 68 patients (mean age 40.3 ± 13.3 years (SD)) with a history of asthma, presented to the Department for Dermatology, Venerology & Allergology at the University hospital of Leipzig, were collected. For control, we obtained 66 serum samples from a healthy control cohort (mean age 42.8 ± 13.4 years (SD)) (non-asthmatics) accrued from the Leipzig Research Centre for Civilization Diseases-LIFE. The age and gender distribution is shown in Figure 1. There were no statistically significant differences in the average age or gender distribution between the asthmatic and control cohort.

Asthma severity according to Global Initiative for Asthma (GINA) classification was as follows: 49 patients had mild asthma (grade 1), 13 patients grade 2, and 6 patients grade 3. Therapy in asthma patients at time of investigation was as follows: all had inhalative treatment with betamimetics ($n = 36$ at least once daily), $n = 32$ had inhalative glucocorticosteroids, $n = 15$ had antihistamines and $n = 4$ Montelukast®. None of them received systemic glucocorticosteroids at time of investigation.

Total serum IgE levels and blood eosinophils

The serum of 68 asthmatics and 66 non-asthmatics had been subjected to measurement of serum IgE levels. The median peak serum IgE levels for asthmatics was 257 kU/l (IQR: 84–2,572 kU/l) (Fig. 2A). Eleven asthmatics had IgE levels higher than 5,000 kU/l (laboratory detection limit)

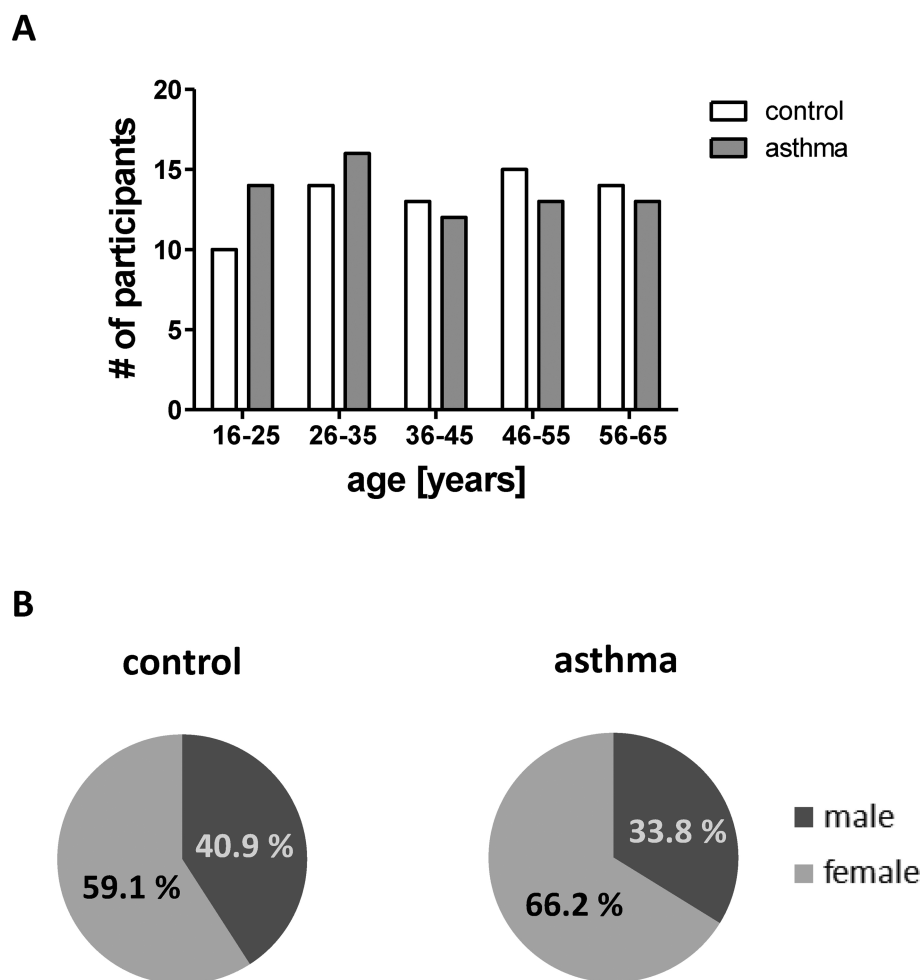


Figure 1. Age and gender distribution of asthmatics and non-asthmatics. Serum samples obtained from 66 healthy controls (white, accrued by LIFE) and 68 asthmatics (grey, accrued from Department for Dermatology, Venerology & Allergology), with a severity from 1 to 3, were used in the study presented here. Participants were separated in decade age-groups ranging from 16 to 65 years (A). The gender distribution is shown for controls and asthmatics separately (B).

and were assumed as 5,000 kU/l for further evaluation. Among the control cohort, the median serum IgE level was significantly lower than in the asthmatics group (i.e., 38.9 kU/l (IQR: 17.2–85.9 kU/l)). No significant differences were found in the frequency of eosinophils in the blood of asthmatics (60 tested) as compared with non-asthmatics (66 tested) (Fig. 2B).

Immunoglobulin G reactivity against

C. neoformans

In prior published studies antibodies directed against *C. neoformans* were detected by ELISA, FACS, and Western Blot [12,30,31]. In our study we decided to choose flow cytometry (FACS) for serological screening to test for the presence of *C. neoformans*-specific IgG antibodies. Using the CAP67 strain of *C. neoformans* which lacks a capsule [29] allowed for serotype-independent detection of *C. neoformans*-specific IgG.

To test for the presence of fungus-specific IgG antibodies *C. neoformans* CAP67 cells were incubated with purified rabbit anti-*C. neoformans* IgG as a positive control (Fig. 3A), with sera from intranasally infected mice as an additional control (Fig. 3B) or naïve mice (data not shown), or with serum from asthmatics and non-asthmatics (Fig. 3C). As a negative control cells were incubated with the respective isotype control followed by a fluorochrome-labeled detection antibody (Fig. 3A) or only with the secondary fluorochrome-labeled antibody conjugate (Fig. 3B, C). In earlier experiments the peak of mean fluorescence intensity was reached at serum dilutions between 1:100 and 1:200 of eight randomly selected sera from asthmatics (data not shown). Therefore, all serum samples were used at a 1:200 dilution. After exclusion of dead cells and doublets (Fig. 3A–C) the geometric mean of fluorescence intensity of each sample was recorded. As expected, incubation of *C. neoformans* CAP67 with purified rabbit

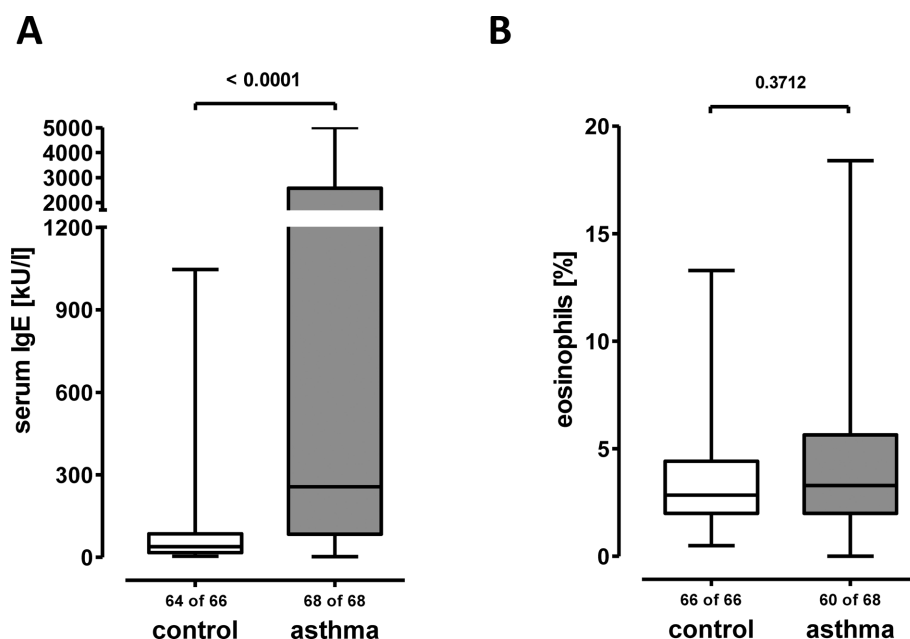


Figure 2. Serum IgE concentration and frequency of eosinophils in the blood of asthmatics and healthy controls. The determination of total serum IgE concentration (A) and frequency of eosinophils (B): IgE concentrations above 5,000 kU/l were considered as 5,000 kU/l. Statistical analysis was done using the nonparametric Mann–Whitney test.

anti-*C. neoformans* IgG as a positive control resulted in a significant shift in the MFI (Fig. 3A). Moreover, upon incubation with serum from experimentally infected mice (used as an additional positive control) a similar shift in the MFI was observable (Fig. 3B). Sera from naïve mice showed no unspecific antibody binding to cryptococcal antigens (data not shown). Finally, human sera of healthy individuals or asthmatics were analyzed. A representative sample from the asthma cohort shown in Figure 3C demonstrates a shift in MFI which, interestingly, is comparable to the positive control sera from an immunized rabbit (Fig. 3A) and from infected mice (Fig. 3B). As we have analyzed serum samples from patients at an age starting from 17 years, we lack human sera that are definitively negative for IgG antibodies against *C. neoformans*. Even cord blood, which contains maternal IgG, seems to be positive for *C. neoformans*-specific antibodies [14]. For this reason sera leading to a MFI smaller than the MFI (plus the three-fold standard deviation) from *C. neoformans* CAP67 cells that had been incubated with the fluorochrome-labelled secondary antibody alone, were considered to be negative for IgG antibodies against *C. neoformans*. In fact, only three sera from the asthmatics cohort were not considered for further analysis. When comparing sera from asthmatics with sera from healthy controls only a small but not significant increase in the specific IgG reactivity was found in asthmatics (Fig. 3D). No differences in staining intensity of cryptococci were observed when sera of patients with

different asthma severity grades were compared. However, the majority of patients studied belonged to grade 1 in the GINA classification. Furthermore, there is no correlation between total serum IgE levels and fungus-specific IgG (data not shown). To confirm the *C. neoformans* specificity of serum samples we preabsorbed 2 high, 4 medium, and 2 low reactive sera from both asthmatics and healthy controls with two different *C. neoformans* strains (CAP67 and H99) and a *Candida albicans* strain (SC5314). Sera without preabsorption were used as control and the reduction of fluorescence intensity after preabsorption was calculated. Serum samples that had been preabsorbed with two different *C. neoformans* strains prior to the detection of anti-*C. neoformans* antibodies showed a significant reduction in MFI compared to serum preabsorbed with *C. albicans* (Fig. 3E) pointing to the specificity of the detected IgG for *C. neoformans*. The different capability in reduction of MFI of both *C. neoformans* strains is most likely due to the intact polysaccharide capsule of strain H99 that may cover protein structures otherwise detectable on strain CAP67. Another reason may be serotype-specific capsular differences. In addition to FACS analysis, the presence of *C. neoformans*-specific antibodies was also tested by ELISA using *C. neoformans* CAP67 protein extracts. There was a significant correlation between reactivity against *C. neoformans* CAP67 by FACS (MFI) and ELISA (OD) in both healthy controls (Fig. 4A, $r_s = 0.42$, $P < .001$) and asthmatics (Fig. 4B, $r_s = 0.32$, $P = .009$). Therefore, and due to

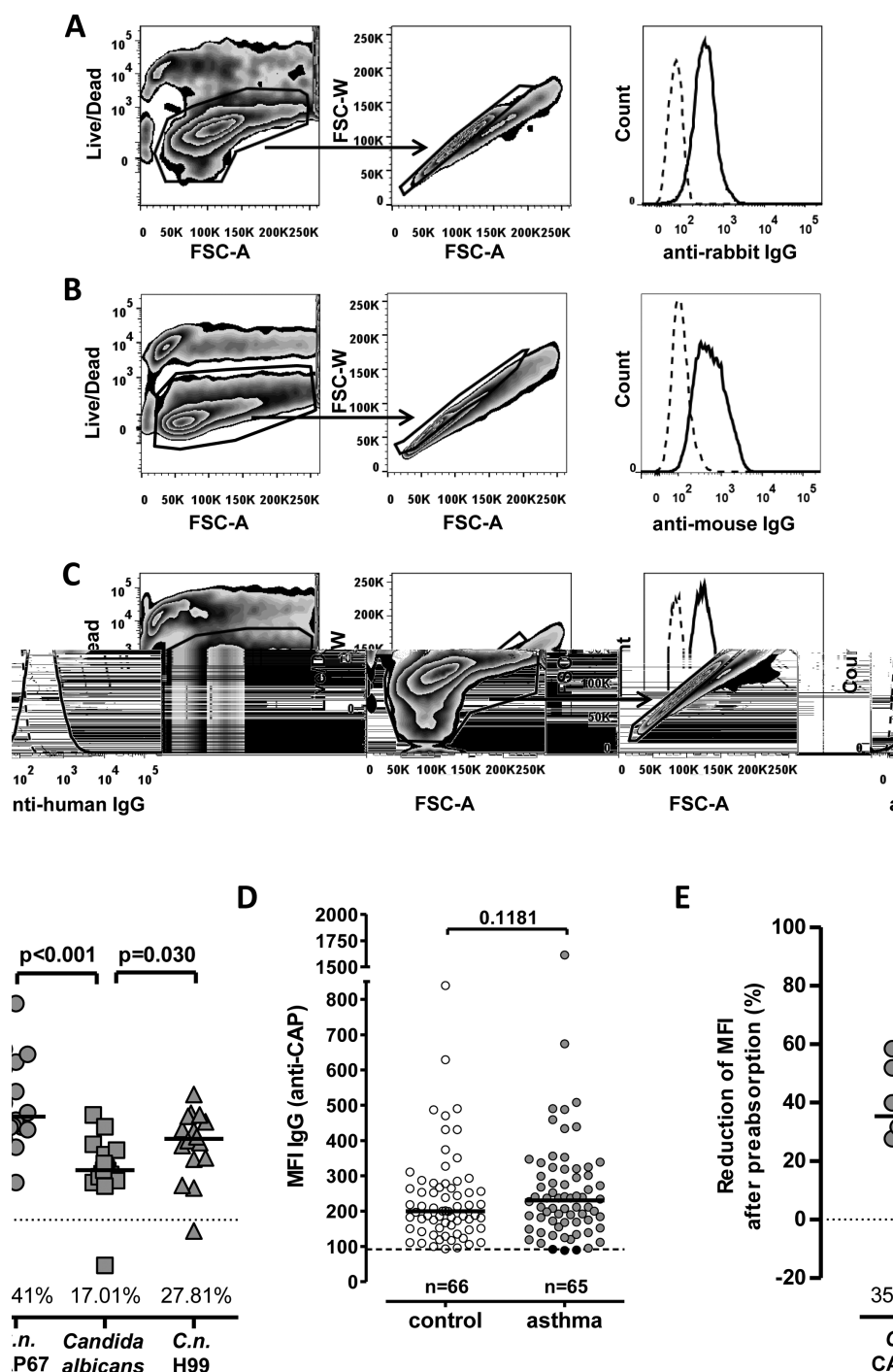


Figure 3. Determination of specific IgG reactivity against *Cryptococcus neoformans* in human serum samples. 1×10^6 cells of *C. neoformans* CAP67 were incubated with a live dead staining dye. After washing, cells were incubated with 0.5 μ g rabbit anti-*C. neoformans* IgG (A) or mouse (B) and human serum samples (dilution 1:200) (C). Rabbit, mouse, and human IgG were detected using Alexa Fluor[®] 488-labelled anti-rabbit IgG, FITC-labelled rat anti-mouse IgG, or FITC-labelled goat anti-human IgG antibody, respectively. After exclusion of dead cells (A-C left) and doublets (A-C middle), single cells that had been incubated with rabbit anti-*C. neoformans* IgG, mouse, or human serum and the respective detection antibody (bold line) or with the isotype control/secondary antibody alone (dashed line) were analysed for fluorescence intensity (A-C right). Representative density plots and histograms from the asthma cohort are shown (C). D) The geometric mean fluorescence intensities (MFI) obtained from serum samples of healthy controls (white) and asthmatics (grey) are compared. The dashed line indicates the arbitrarily defined threshold level (i.e., MFI of cells incubated with secondary antibody alone + $3 \times$ SD). Black data points were below this threshold and not analysed. Statistical analysis was done using the nonparametric Mann-Whitney test. E) Serum samples (dilution 1:200, sera from control and asthma group) were incubated with or without 4×10^6 cells of *C. neoformans* (CAP67, H99) or *Candida albicans*. Following centrifugation supernatants were applied to 1×10^6 cells of *C. neoformans* CAP67 and bound antibodies were visualized using the secondary antibody. Depicted is the relative difference of MFI of untreated vs. preabsorbed sera. Statistical analysis was done using the Mann-Whitney test. Percentages indicate the medians.

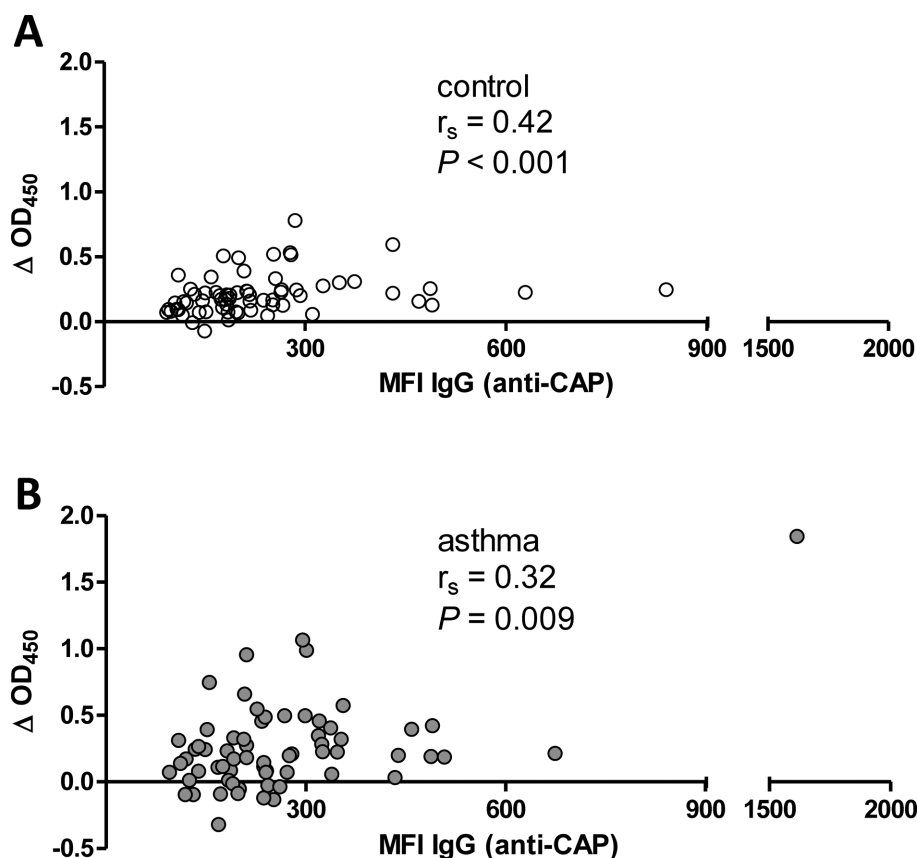


Figure 4. Correlation between antibody titers determined by ELISA or FACS analysis. Serum samples from healthy controls (A) or asthmatics (B) were analysed for seroreactivity against *C. neoformans* (CAP67) by ELISA (ΔOD_{450}) or FACS analysis (MFI IgG). Spearman's rank correlation coefficient (r_s) was calculated and there is a significant correlation in seroreactivity determined by ELISA or FACS analysis in both healthy controls ($P < .001$) and asthmatics ($P = .009$).

the higher sensitivity of flow cytometry, mainly the results of FACS analysis are shown here.

It was reported that males with cryptococcosis are significantly higher affected as females with cryptococcosis as measured by fungaemia, disseminated infection and mean serum antigen titer [32]. To take this into account we compared the IgG reactivity of male versus female participants in both asthmatics and control cohort. The results shown in Figure 5 demonstrate that male asthmatics have significantly higher IgG antibody levels compared to female asthmatics ($P = .044$). Of note, the serum with the highest content of IgG antibodies (MFI: 1,613) against *C. neoformans* belongs to the female asthma group. Excluding this sample from the analysis increases the statistical difference between both groups ($P = .027$). Furthermore, comparing male asthmatics with healthy males an increased seroreactivity in male asthmatics is detected at the border of significance ($P = .07$). This indicates a higher prevalence of subclinical infections with *C. neoformans* in male asthmatics. Significant differences in seroreactivity were neither observed between males and females of the control group nor between female asthmatics and non-asthmatics. No

significant differences could be observed when comparing asthmatics with non-asthmatics in different age groups (data not shown).

Discussion

Asthma is a chronic allergic inflammatory disease and sensitization to fungal allergens is associated with severity of asthma [33,34]. The explorative study described here represents an important initial analysis of seroreactivity against *Cryptococcus neoformans* which indicates (i) a high frequency of cryptococcal seroreactivity within the population of a restricted urban area in Germany (i.e., 131 seropositive sera out of 134 in total), (ii) only a low level of antibody reactivity of individual sera albeit of comparable magnitude to experimentally infected mice, and (iii) a trend of a higher titer in the asthma group. This important finding should be further validated in a bigger cohort (i.e., 500 individuals) as revealed on the basis of our statistical calculations. A future study should include individuals from rural areas to allow for a comparison between different environments.

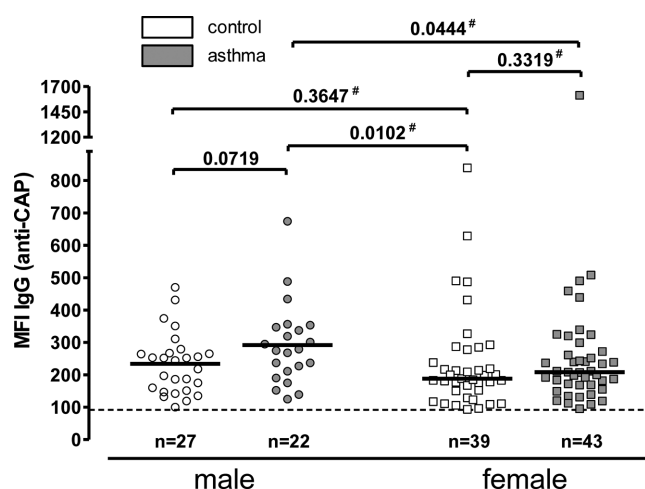


Figure 5. Gender-dependent specific IgG reactivity against *Cryptococcus neoformans* in serum from asthmatics and non-asthmatics. Serum samples (dilution 1:200) from female (squares) and male (circles) asthmatics (grey) as well as non-asthmatics (white) were analysed for the presence of IgG antibodies against *C. neoformans* CAP67. Shown are the geometric mean fluorescence intensities. Each value represents reactivity of an individual serum sample. Statistical analysis was done using the nonparametric Mann–Whitney test (#) or unpaired Student's *t*-test. Dashed line indicates the arbitrarily defined threshold level (i.e., MFI of cells incubated with secondary antibody alone + $3 \times \text{SD}$).

Despite the minor differences observed between the two groups we compared the putative influence of gender of both groups on *C. neoformans*-specific IgG. Indeed, there is a higher seroreactivity against the fungus in male asthmatics compared to female controls and female asthmatics. Albeit asthma is clearly different from invasive mycosis (such as cryptococcosis) this observation seems to be similar to previous epidemiological studies that observed a higher incidence of cryptococcosis in male patients than in female patients before as well as after the start of human immunodeficiency virus (HIV) epidemic [35–38]. Therefore, it is tempting to speculate that subclinical or persistent infection preferentially occurs in male individuals leading to increased humoral responses.

Furthermore, *in vitro* studies using monocyte-derived macrophages from males and females showed a higher phagocytic uptake of fungal cells, a lower intracellular fungal burden, and lower macrophage killing by *C. neoformans* in macrophages from female donors compared to their male counterparts [39]. Even in mice the fungal organ burden was higher in males than in females supporting the hypothesis that there is a difference in interaction of the fungus and the host immune system between males and females that results in a less efficient control of infection [39]. This underlines the higher incidence of cryptococcosis in male patients.

Besides using clinical investigation to diagnose asthma, total serum IgE concentration and frequency of blood

eosinophils were determined. Although children with allergic asthma had higher absolute numbers (and frequencies) of blood eosinophils and a positive correlation of total serum IgE levels and blood eosinophils count [40], we could only observe an increase in total serum IgE in asthmatics, whereas the frequency of eosinophils did not differ between the two groups (Fig. 2). Epidemiologic and clinical observations revealed a positive correlation of IgE with the severity of asthma [41] and infiltration of the airway by lymphocytes and eosinophils have been found in patients with mild-to-moderate asthma [42].

Antibodies specific for *C. neoformans* are commonly detectable in both immunocompetent and immunosuppressed individuals [12,30,43]. Information about the glucuronoxylomannan (GXM) serotypes from *C. neoformans* endemic for the Leipzig area is not available. Thus we decided to screen seroreactivity against the acapsular *C. neoformans* strain CAP67 to include all cryptococcal serotypes potentially present in this area. When comparing asthma and control groups no significant differences in mean fluorescence intensities are detectable. We found only a tendency toward a higher IgG-mediated seroreactivity in asthmatics. Nevertheless, this is similar to a recent study where *C. neoformans*-specific immunoglobulins were detected in the bronchoalveolar lavage fluid (BALF) of asthmatic and non-asthmatic children. The authors found also a trend toward increased IgG reactivity to *C. neoformans*, whereas the IgA reactivity was significantly increased in asthmatics [31]. As there was no clinical indication for bronchoscopy in our study, BALF was not available for analysis. Thus, we have only analyzed serum for fungal-specific antibodies where IgA-mediated reactivity is not to be expected. Furthermore, Goldman *et al.* detected fungal-specific antibodies in the BALF of children with severe asthma^[31], whereas in our study adults, most of them with a mild form of asthma (most of them with grade 1 according to the GINA classification), were analyzed.

In summary, we describe a high frequency of seroreactivity against *C. neoformans* among people that live in an urban area in Germany. There is a trend toward increased *C. neoformans*-specific IgG titers in the serum of asthmatics compared with the non-asthmatic control. Among asthmatics, the highest reactivity is observed in males. This initial study helps to design future bigger surveys that deal with modern civilization diseases to understand the contribution of fungal antigens in the context of asthma.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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