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Detection of anti-*Histoplasma capsulatum* antibodies and seroconversion patterns in critically ill patients with COVID-19: An underdiagnosed fungal entity complicating COVID-19?

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Abstract

The patients with severe COVID-19 are at increased risk for invasive fungal infections, such as invasive pulmonary aspergillosis and candidiasis, which increase morbidity and mortality. However, clinicians should also consider the possibility of reactivating latent *Histoplasma capsulatum* in patients with severe COVID-19 living within areas of endemicity who have worsening respiratory function or sepsis, even if they do not have classical risk factors for histoplasmosis (e.g., HIV/AIDS). Bearing in mind this scenario, serum samples of 39 non-HIV/AIDS patients from Buenos Aires hospitalized due to severe COVID-19 pneumonia were analyzed for anti-*H. capsulatum*-specific IgG antibodies by an in-house ELISA. Antibodies against *H. capsulatum* were detected in the sera of 8/39 patients (20.51%). To exclude the possibility that these antibodies arose from past exposure of these patients to the fungus, paired serum samples obtained after an interval of at least 10 days were evaluated. Of them, five patients (62.5%) with negative anti-*H. capsulatum* antibodies at baseline became seropositive 7–10 days later. Three patients (37.5%) had positive anti-*H. capsulatum* antibodies at baseline, but at time point 2, one of them became seronegative and the other one diminished the antibody titers (4000 vs. 16000 at baseline). The remaining patients displayed higher antibody titers at *time point* 2 (4000 vs. 1000 at baseline) and died immediately thereafter. In conclusion, awareness of the possibility of fungal co-infections is essential to reduce delays in diagnosis and treatment in order to help prevent severe illness and death from these infections.

Lay summary

This study verifies that patients with severe COVID-19 at ICU are at risk for histoplasmosis reactivation in endemic areas. Accurate diagnosis of this deadly fungal disease among critically ill patients with COVID-19 living in endemic areas for histoplasmosis is needed.

Key words: SARS-CoV-2, Histoplasmosis, Seroconversion, Severe respiratory failure, COVID-19-associated mycoses.

Introduction

Histoplasmosis is a disease caused by the thermodimorphic fungus *Histoplasma capsulatum* that is widely distributed in many areas of North, Central and South America, Africa, Asia, and Australia.[1] Although generally harmless to an immunocompetent host, the infection can be deadly in patients with an impaired immune system, such as those with HIV/AIDS, or in individuals receiving steroids or other immunosuppressants, which leads to a defect in the T lymphocyte-macrophage response to *H. capsulatum*.[2] In this cell-mediated immunosuppressed population, histoplasmosis may occur either after direct exposure to the fungal spores or after reactivation of a latent focus of infection acquired years before.[3] In Argentina, this disease is endemic but its prevalence is underestimated due to the lack of epidemiological studies and notification. However, even before the COVID-19 pandemic, its prevalence was high among immunocompromised individuals, particularly in those with HIV/AIDS, in whom disseminated histoplasmosis was the first AIDS-defining condition in 70% of them.[4]

Noteworthy, histoplasmosis symptoms are nonspecific and may be similar to those of other respiratory diseases, including COVID-19, thus complicating diagnosis and treatment. Accordingly, pulmonary manifestations in histoplasmosis range from asymptomatic infection to diffuse alveolar disease, causing respiratory failure and even death, overlapping these severe symptoms with those described in COVID-19 patients with acute respiratory distress syndrome (ARDS).[5,6]

Furthermore, critically ill patients with COVID-19 who require mechanical ventilation in intensive care units (ICU) are at increased risk for opportunistic infections, which increase morbidity and mortality. Those that have a fungal origin have become especially important and concerning during the COVID-19 pandemic, particularly invasive pulmonary aspergillosis (CAPA) and candidiasis,[7] although other fungal infections were described, including mucormycosis,[8] fusariosis[9] and trichosporinosis.[10] However, the relationship between COVID-19 and systemic endemic mycoses is less clear, with only a few published case reports of coccidiomycosis, histoplasmosis, and paracoccidioidomycosis in COVID-19 patients.[11–13] Particularly, all concomitant COVID-19 associated cases of histoplasmosis were reported among HIV/AIDS patients.[14,15]

However, clinicians should also consider the possibility of reactivating latent *H. capsulatum* in those patients with severe COVID-19 living within areas of endemicity who have worsening respiratory function or sepsis, even if they do not have classical risk factors for histoplasmosis (e.g., HIV/AIDS).

Bearing in mind this scenario, a retrospective study for the detection of anti-H. capsulatum antibodies from serum samples of 39 patients from Buenos Aires (an endemic area for *H. capsulatum*) with moderate-to-severe ARDS due to severe COVID-19 pneumonia was performed.

Methods

Serum samples

Sera from a cohort of 39 patients that were initially sampled to study the presence of *Aspergillus* (serum galactomannan (GM) testing) and that were kept at -80°C for further studies were retrospectively analyzed for anti-*H. capsulatum* antibodies and seroconversion by immunodiffusion and ELISA. All these patients were admitted to the intensive care unit (ICU) of Hospital Posadas with moderate-to-severe ARDS due to severe COVID-19 pneumonia.

Also, serum samples from 22 patients with HIV/AIDS and progressive disseminated histoplasmosis confirmed by culture or histopathology/cytology were studied for the detection of anti-*Histoplasma* antibodies for validation of the in-house ELISA test. Specimens from 20 healthy individuals from an endemic area of histoplasmosis (Buenos Aires) were used to determine the cut-off value.

Measurements of total IgG antibody levels were performed using an automated turbidimetric immunoassay (IgG MonlabTest®, Spain).

Immunodiffusion (ID) assay

ID assays were performed on 50 mm Petri dishes covered with a layer of 1% Noble Agar in isotonic saline solution balanced with phosphates at pH 7.0, plus 6000 polyethylene glycol and phenol 0.3%. The presence of precipitation bands for the antigen-antibody reaction was checked 72 h later, as described previously.[16]

ELISA

An in-house ELISA using a commercially available standardized histoplasmin as reagent was used for detecting anti-*H. capsulatum* antibodies as described by Guimaraes and colleagues[17] with some modifications.[18] Briefly, 96-well flat-bottom microtitre plates (Nunc MaxiSorp™; Thermo Scientific, Denmark) were coated with histoplasmin (IMMY, USA) solution containing 0.1 μg of protein per well diluted in phosphate buffered saline (PBS) pH 7.4 and incubated at 4°C overnight. After blocking with 5% (w/v) dried non-fat milk in PBS containing 0.05% Tween 20 (PBST), serum samples were added to each well at a dilution of 1:1 000; 1:4 000 and 1:16 000 in PBST/non-fat milk and plates were incubated at room temperature for 1 h. Then, plates were incubated with goat anti-human IgG horseradish peroxidase conjugate (Jackson Immunoresearch Laboratories, USA) diluted 1:10 000 in PBST/non-fat milk. The reaction was developed with o-phenylenediamine dihydrochloride (Thermo Scientific) 2 mg/ml and 0.03% (w/v) H_2O_2 diluted in 0.1 M sodium citrate buffer, pH 5.0. The reaction was terminated by the addition of 4 N H_2SO_4 and absorbances at 492 nm were measured on a microplate reader Multiskan GO™ (Thermo Scientific).

To determine the discriminatory power and accuracy of the in-house ELISA test, ROC (receiver operating characteristic) curves were calculated using the GraphPad Prism version 8.4.2 (679) with data from the ELISA. The cut-off value was obtained as the mean plus three standard deviations (S.D.) from sera from healthy individuals living in an endemic area of histoplasmosis. Sera from individuals with confirmed histoplasmosis were classified as true positive, TP, when they presented O.D. values above the cutoff in the serological test or as false negative, FN, when they presented O.D. values below the cutoff. Sera from healthy control individuals were classified as true negative, TN, when they presented O.D. values below the cutoff in the serological test or as false positive, FN, when they presented O.D. values above the cutoff. The parameters sensitivity, specificity, positive and negative predictive values and global efficiency were obtained with the following formulas: sensitivity = TP/(TP + FN); specificity = TN/(TN + FP); positive predictive value = TP/(TP + FP); negative predictive value = TN/(TN + FN); global efficiency = TP + TN/TP + TN + FP + FN. 95% Confident intervals (95%CI) were calculated for the assay sensitivity and specificity.

Histoplasma Galactomannan enzyme immunoassays

Three urine samples recovered from two patients that were seroreactive for anti-*H. capsulatum* antibodies were tested using the *Clarus Histoplasma Galactomannan Enzyme Immunoassay*® (IMMY, USA) according to the manufacturer's instructions. Although the use of this kit is described only for urine samples, eight serum samples from the patients that were seroractive for anti-H. capsulatum antibodies were also analyzed following the established protocol for urine after EDTA/heat pretreatment as described elsewhere.[19]

Statistical analysis

Analyses of patient demographics and baseline characteristics among the different COVID-19 groups *(H. capsulatum* seronegative vs. *H. capsulatum* seropositive) were conducted using the InfoStat Statistical Software Version 2020 for Windows. Numerical variables with non-normal distribution were compared using the Wilcoxon test and categorical variables were compared using the Pearson´s $\chi 2$ test. *P*-values < 0.05 were considered significant.

Ethical considerations

This study was approved by the institutional review committee 'Dr Vicente Federico Del Giúdice' at Hospital Nacional Alejandro Posadas, Buenos Aires, Argentina (Ref. 395 EMnPES0/20).

Results

Performance of the antibodies detection ELISA using histoplasmin

The mean O.D of healthy individual sera was 0.140 ± 0.113 and the median was 0.104, resulting in a cut-off value of 0.339. The mean O.D. of sera from individuals with histoplasmosis was 1.109 ± 0.648 and the median was 1.347. This ELISA had a sensitivity of 82% (18/22 cases; 95% CI: 66–98) in detecting confirmed disseminated histoplasmosis cases and a specificity of 90% (18/20 controls; 95% CI: 77-1). The positive and negative predictive values were 90 and 82%, respectively, and the global efficiency was 86%. The AUC was 0.9045 ± 0.0009 (95% CI: 0.80–1.00) (Fig. 1).

ID and ELISA tests for anti-*H. capsulatum* antibodies detection in severe COVID-19 patients

Antibodies against the H and M glycoproteins of a commercially available standardized histoplasmin were undetected by ID assays in all cases (0/39). However, antibodies against histoplasmin were detected by ELISA in the sera of 8/39 patients (20.51%). To exclude the possibility that these antibodies arose from past exposure of these patients to the fungus, paired serum samples obtained after an interval of at least 10 days were evaluated. As shown in Fig. 2, five patients (P3, P4, P5, P6, P7) with negative *H. capsulatum* specific IgG antibodies at baseline (time point 1) became seropositive 7–10 days later (time point 2) and four of them (P3, P5, P6, P7) died shortly after. Noteworthy, patients P3 and P7 presented higher antibody titers in a third time point.

Three patients (P1, P2, P8) presented positive *H. capsulatum*-specific IgG antibodies at baseline (time point 1). Later at time point 2, P8 became seronegative and P1 diminished the antibody titers (4 000 vs. 16 000 at baseline) and both were discharged from hospital a few days later. However, the remaining patient (P2) displayed higher antibody titers in the second time point (4 000 vs. 1 000 at baseline) and died immediately thereafter.

Total serum IgG were within normal ranges (700–1600 mg/dl) in all cases, except for three samples that presented slightly elevated total IgG values. However, in the two patients in whom total IgG values were slightly elevated, these values remained relatively constant over time despite the increase in the titer of anti-*Histoplasma* antibodies. The titers of total IgG and specific anti-*Histoplasma* IgG antibodies are depicted in Fig. 3. As it is shown in Table 1, patients that were seroreactive for *Histoplasma* infection were predominantly male (87.5%) and had a slightly higher median age (63.2 vs. 51.5) than those non-seroreactive for this fungal infection. In addition, patients with detectable anti-*Histoplasma* antibodies had more underlying diseases, which mainly included hypertension (87.5 vs. 53.3%), diabetes (75.0 vs. 36.6%) and obesity (62.5 vs. 43.3%), as well as higher mortality (62.5 vs. 34.4%) compared to those patients

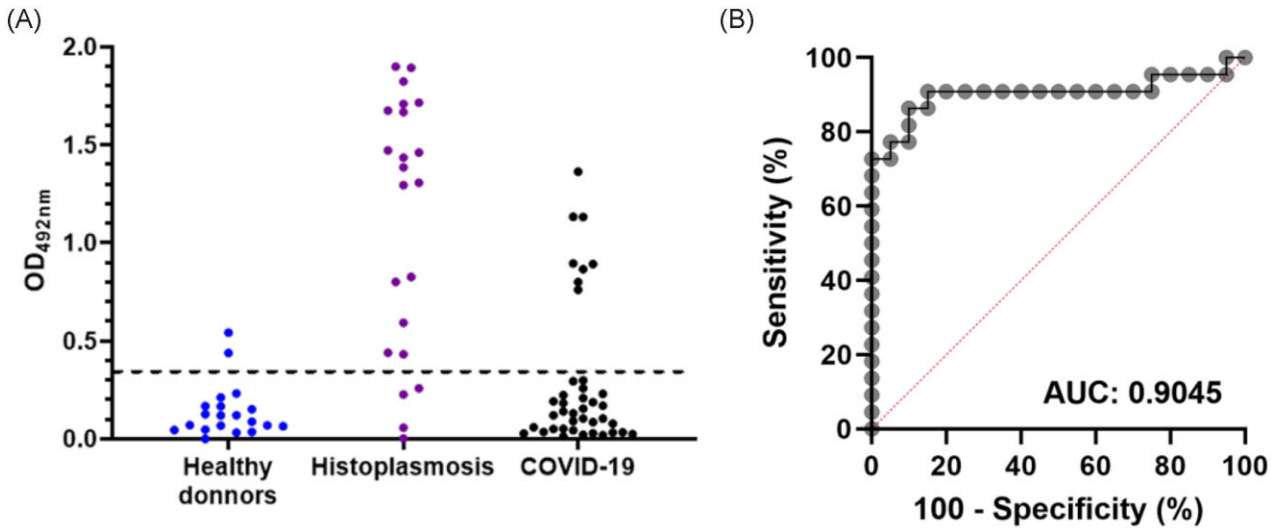

Figure 1. Results of the in-house ELISA using histoplasmin as reagent. (A) O.D. obtained from the sera of healthy donors, individuals with confirmed histoplasmosis and the 39 COVID-19 studied patients. Horizontal dashed-bars (- - -) represent the cutoff. (B) Receiver operating characteristics (ROC) curve for histoplasmin. The ROC is plotted between the true-positive rate (sensitivity) on the *y*-axis, and the false-positive rate (1-specificity) on the *x*-axis. The area under the curve (AUC) represents the accuracy of the ELISA test, which was 0.9045.

without detectable anti-*H. capsulatum* antibodies. Although these differences were not statistically significant among each group, except for hypertension.

The median duration of hospitalization at UCI were statistically significantly higher in those seroreactive *Histoplasma* patients (83.6 vs. 46 days, <0.05, Wilcoxon test), and mechanical ventilation was slightly more frequent in the former group than in the later (87.5 vs. 77.4%).

Histoplasma galactomannan antigen was detected in the urine of patients P2 and P8. On the other hand, *Histoplasma* galactomannan detection in sera was positive in patients P6 and P7 according to the cut-off criteria of the kit *Clarus Histoplasma Galactomanan EIA*® (EIA units ≥1.00 are considered positive in urine samples). A summary of these results is presented in Table 2.

Aspergillus galactomannan testing using the *Platelia Aspergillus* kit (Bio-Rad) was performed in all serum samples as a complementary tool for detecting COVID-19 associated pulmonary aspergillosis. All samples tested negative for *Aspergillus* GM.

Discussion

Patients with severe COVID-19 at ICU are susceptible to many microbial coinfections, including those caused by opportunistic fungi.

As overburdened hospitals attempt to identify and care those patients afflicted with COVID-19, it is easy to overlook endemic diseases that might mimic the pulmonary manifestations of COVID-19, and even may reactivate or co-infect those weakened by COVID-19, exacerbating their clinical condition.

In endemic areas for systemic mycoses, such as histoplasmosis, clinicians should suspect reactivation of an old infection (primoinfection). Since patients with severe COVID-19 might have an impaired ability to mount a humoral immune response, either related to the immune dysfunction caused by SARS-CoV-2 or to the immunosuppressants used at ICU (e.g., corticosteroids), the detection of specific fungal antibodies by immunodiffusion might be limited, as it occurs with HIV/AIDS patients with disseminated histoplasmosis.[17,20] Therefore, a negative result obtained by immunodiffusion does not rule out the possibility of histoplasmosis. In this regard, histoplasmosis diagnosis in severe COVID-19 patients can be improved through the use of paired serum diagnostic algorithm by a more sensitive technique such as ELISA. However, the only antibodies detection ELISA cleared by the Clinical Laboratory Improvement Amendments is a fee-for-service test offered by MiraVista Diagnostics in Indianapolis, which it is not a feasible option for laboratories outside the USA. Nevertheless, several in-house ELISA were developed using histoplasmin as the antigen with considerably good results.[17,18,21–26]

The overall sensitivity of our in-house antibody detection ELSIA using a commercial standardized histoplasmin was 82% (18/22 cases; 95% CI: 66–98) among confirmed immunosuppressed patients with disseminated histoplasmosis and the specificity was 90% (18/20 controls; 95% CI: 77-1). Using this ELISA, we found that 20.51% (8/39) of the COVID-19 patients were seroreactive for *H. capsulatum*. Although the presence of anti-*Histoplasma* antibodies is suggestive of active *Histoplasma* infection, it may also represent previous exposure to fungus since these antibodies may persist for years after infection.[27] However, a positive result in a patient with compatible clinical symptoms,

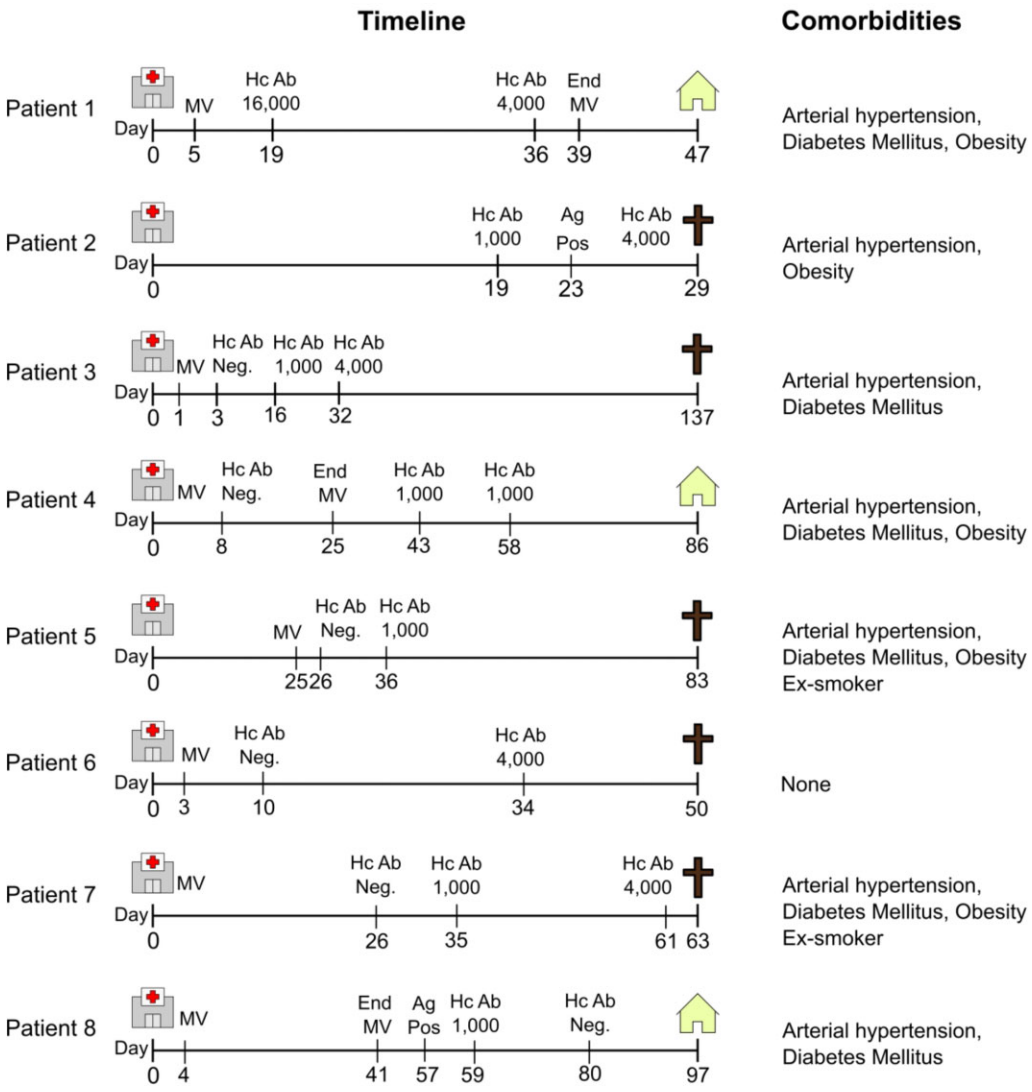

Figure 2. Timeline showing the clinical evolution of the eight patients that were seroreactive for anti-*H. capsulatum* antibodies.

risk factors (i.e., therapy with corticoisteroids) and resident of an endemic area represents a scenario that deserves further testing. Hence, seroconversion or a 4-fold increase in titers was analyzed in search for more suggestive evidence of histoplasmosis. Bearing this in mind, only about 6 out of 39 (15.4%, P2-P7) of the patients have serological evidence that is suggestive of an active infection. Despite the lack of serological evidence in P8, the *Histoplasma* antigen detection in urine also suggests a probable infection.

Antigen detection in histoplasmosis is largely used in disseminated forms of the disease in immunosuppressed patients. Unfortunately, the currently available specific *Histoplasma* antigen detection assay in our country (Clarus *Histoplasma* Galactomannan EIA, IMMY) is validated only for urine samples. In the present study, two patients (P2 and P8) had a positive urinary *Histoplasma* antigen test, out of the eight *Histoplasma*-seroreactive patients. In addition, patients P3 and P4 patients presented detectable galactomannan levels ≥1.00 EIA units in serum using the Clarus *Histoplasma* assay. Since Buenos Aires is an endemic area for histoplasmosis and one of the most cities affected by SARS-CoV-2 in Argentina, we suppose that the cases presented herein are the tip of the iceberg of the real magnitude of this problem. An active prospective search of similar cases is thus necessary.

Noteworthy, all patients that rendered positive anti-*H. capsulatum* antibodies were non-HIV/AIDS patients that received broad-spectrum antibiotics, corticosteroids (8 mg/day dexamethasone), and required oxygen support or mechanical ventilation due to moderate-to-severe ARDS. All patients except one were male with a median age of 66 years (range 51–74) and have ≥1 underlying comorbidity such as hypertension, diabetes and obesity. Two of them were ex-smokers. None of them received antifungal drugs.

Because of the severe immunomodulation and lymphocyte depletion caused by the SARS-CoV-2 itself and the subsequent administration of certain drugs directed at the immune system (i.e.,

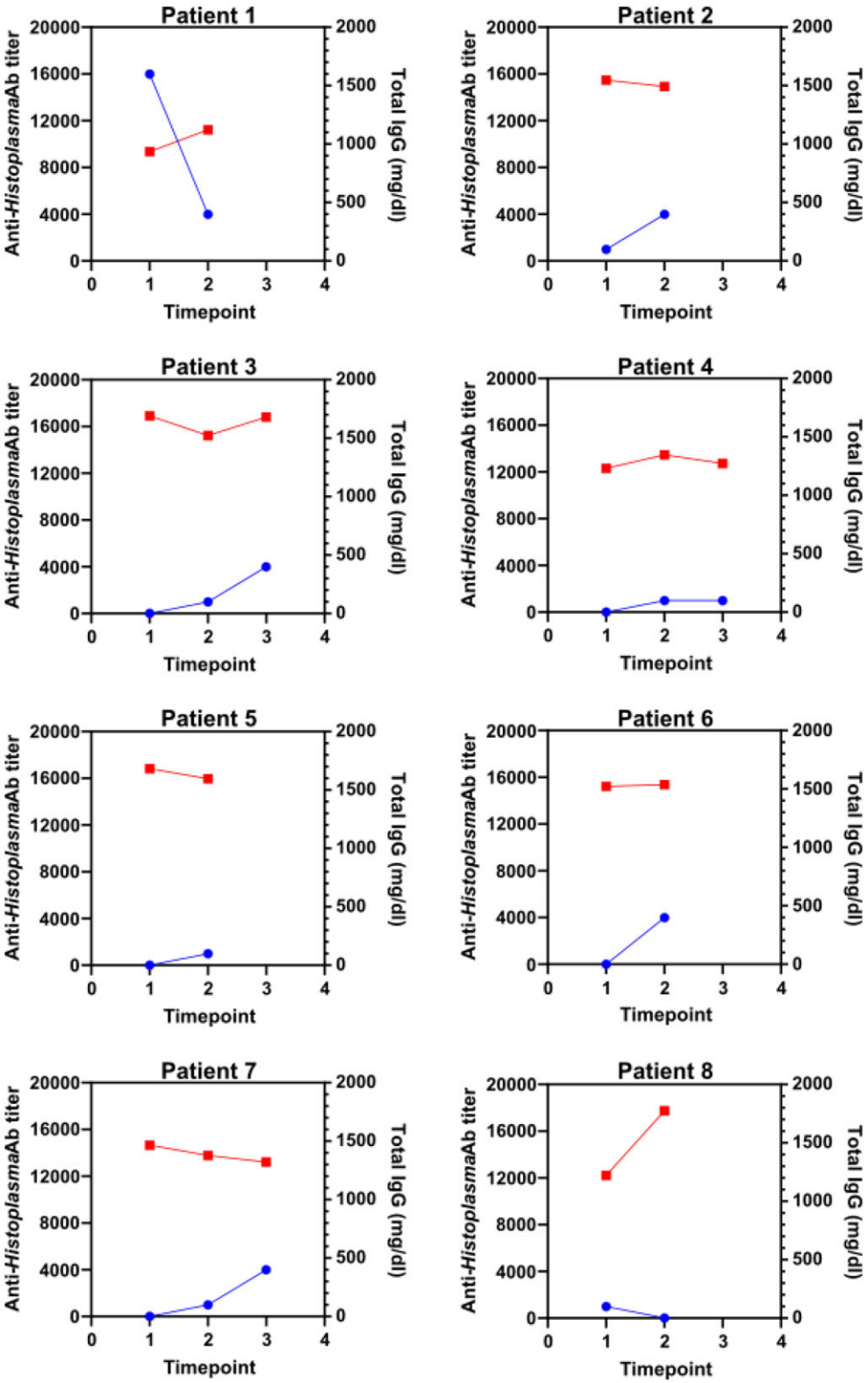

Figure 3. Titers of anti-*Histoplasma* IgG antibodies (blue circles) relative to total IgG (orange squares) in the eight patients that were seroreactive for anti-*H. capsulatum* antibodies.

Table 1. Characteristics of the subjects of study.

	All (n = 39)	*H. capsulatum* Seronegative (n = 31)	*H. capsulatum* Seropositive (n = 8)	*P*-value
Age - Years†	59 (19–77)	55 (19–77)	66 (51–74)	0.38
Male	23	16	7	
Female	16	15	1	
Hospitalization - Days†	50 (13–144)	41 (13–91)	77 (30–144)	<0.05
Mechanical ventilation	79.5% (n = 31)	77.4% (n = 24)	87.5% (n = 7)	0.95
Deaths	61.5% (n = 24)	61.2% (n = 19)	62.5% (n = 5)	0.87
Comorbidity				
Obesity	43.6% (n = 17)	38.7% (n = 12)	62.5% (n = 5)	0.28
Diabetes mellitus	43.6% (n = 17)	35.5% (n = 11)	75.0% (n = 6)	0.06
Arterial hypertension	51.3% (n = 20)	41.9% (n = 13)	87.5% (n = 7)	<0.05
Asthma	7.7% (n = 3)	9.7% (n = 3)	0% (n = 0)	0.45
LLA	7.7% (n = 3)	9.7% (n = 3)	0% (n = 0)	0.45
LMA	7.7% (n = 3)	9.7% (n = 3)	0% (n = 0)	0.45
Hypothyroidism	7.7% (n = 3)	6.5% (n = 2)	12.5% (n = 1)	0.32

†Median (range)

corticosteroids),[28] these patients with severe COVID-19, are indeed severe immunosuppressed patients that constitutes a high-risk population for opportunistic fungal infections or reactivations, including those endemic mycoses, such as histoplasmosis.

Table 2. *Histoplasma* Galactomannan results of the *Histoplasma* seroreactive patients.

		Histoplasma GM Ag (EIA units)	
Patient	Timepoint	Urine	Serum
1	T1	NA	0.00
	T2	NA	IS
2	T1	NA	0.00
	T2-6d	3.09	NA
	T2	NA	IS
3	T1	NA	IS
	T2	NA	IS
	T3	NA	0.00
4	T1	NA	IS
	T2	NA	IS
	T3	NA	0.00
5	T1	NA	IS
	T2	NA	0.00
6	T1	NA	IS
	T2	NA	1.47
7	T1	NA	1.62
	T2	NA	IS
	T3	NA	IS
8	T1-2d	1.04	NA
	T1	NA	0.05
	T2	0.91	IS

IS: Insufficient amount of sample; NA: Not available.

In immunosuppressed individuals, histoplasmosis can present with prominent pulmonary manifestations, despite that more often the clinical picture is that of a disseminated infection. Patients who have only pulmonary histoplasmosis have symptoms and signs that are typical for any community-acquired pneumonia, including COVID-19 pneumonia. However, progression to more extensive pneumonia with marked hypoxemia and ARDS can occur quickly in immunosuppressed patients.[5]

Our study, however, has some limitations inherent to retrospective analyses. A definitive diagnosis by culture or histopathology could not be achieved due to the lack of an appropriate sample, as well as a probable diagnosis by the detection of *Histoplasma* urine antigen for the many patients. Only three urine samples from 2 seroreactive patients for *Histoplasma* infection could be recovered. Detection of *H. capsulatum* antigen in bronchoalveolar lavage fluid (BALF) may have been particularly helpful in patients with acute pulmonary histoplasmosis or disseminated disease with pulmonary involvement;[5] however, BALF samples were not available for antigen testing for any patient.

In conclusion, this small cohort study verifies that patients with severe COVID-19 at ICU are at risk for histoplasmosis reactivation in endemic areas. Our findings support the need for accurate diagnosis of this deadly fungal disease among critically ill patients with COVID-19 living in endemic areas for this dimorphic neglected fungus of the America´s. Despite *H. capsulatum* is in the top of the list of AIDS-defining illnesses and AIDS-related deaths in South America, is still mostly undiagnosed in this HIV/AIDS population. And in the COVID-19 population might be most likely undiagnosed since it is not even present on the diagnostic algorithm of a large proportion of clinicians facing a febrile, severely ill COVID-19 patient with a weakened immune status and worsening respiratory function that comes

from and/or live in an endemic area of histoplasmosis. What is not sought, is not found.

As the global COVID-19 pandemic continues worldwide, new challenges arise in the clinical landscape. The high risk of fungal co-infections, including endemic mycoses, is a major threat in severally ill patients with COVID-19 at ICU, but also after recent recovery of COVID-19 pneumonia.[11,29,30]

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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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