The Use of Chemokines and Soluble TNF-α Receptors to Evaluate Patients Treated for Paracoccidioidomycosis

Lilian da Silva Santos1,3*, Weverton César Siqueira2, Samuel Gonçalves da Cruz2, Camila Cristiane Silva Cameo2, Valdirenne Silva Siqueira2, Carolina Venâncio Barbosa2, Alexandre Alvim Ambrósio1, Ana Carla de Carvalho Dantônio1, Alfredo Miranda de Goes1 and Énio Pietra Pedros1,2

1Programa de Pós-graduação em Ciências da Saúde: Infectologia e Medicina Tropical, Faculdade de Medicina, Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil
2Fundação de Medicina, UFMG, Belo Horizonte, Minas Gerais, Brazil
3Laboratório de Imunologia Celular e Molecular, Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, UFMG, Belo Horizonte, Minas Gerais, Brazil
4Corresponding author: Lilian da Silva Santos, Service de Médecine Tropicale et Humanitaire, Avenue de Beau-Séjour, 22, Genève – 1206. Université de Genève, Geneva, Switzerland, Tel: +41-0-22 372 96 15; E-mail: lilianufop@yahoo.com.br

Abstract

Paracoccidioidomycosis (PCM) is a systemic disease with high prevalence in Brazil and some countries in Latin America. This study aimed to evaluate patients treated for PCM in an endemic area, to verify the use of serological markers in the control of cure of this mycosis. A follow-up study of 42 months was conducted with 26 patients, which had blood samples collected during and after treatment. All measures of serological markers were made by ELISA. The dosage of IgG, sTNF-R1 and sTNF-RII allowed the segregation of majority of patients with PCM from health individuals during almost all the period analyzed. Although, some patients did not present detected levels of IgG and sTNF-R1 at any moment. Concentrations of CCL2 and CCL3 were high during the treatment, with a tendency of decreasing along the time. CCL11 was detected with concentrations below the cut-off point during treatment, with increasing from the moment of its interruption. Concentrations of CCL24 did not change along the period analyzed. CXCL9 presented low concentrations during and after the interruption of treatment, without any association with clinical parameters. The variable concentrations found for all serological markers tested show the insecurity to use these parameters and the need of a continuous search for new markers to evaluate the control of cure of patients treated for PCM.

Keywords: Paracoccidioidomycosis; Control of cure; Serological markers; Soluble receptors of TNF-α; Chemokines

Introduction

Paracoccidioidomycosis (PCM) is a systemic disease caused by a complex group of fungi within the Paracoccidioides genus, formed by four distinct phylogenetic lineages known as PS2, PS3, S1, and Pb01 [1,2]. The infection is acquired by inhalation of the mycelial form of the fungus, which once in the alveoli transform into the yeast infective form [3]. PCM is the most prevalent mycosis in Latin America countries with 80% of cases occurring in Brazil followed by Venezuela, Colombia, Ecuador and Argentina [4]. The most affected group is composed by men from 30 to 50 years old, living and working in rural areas. The number of patients clinically diagnosed with PCM may represent only a small portion of infected individuals [5]. Endemic areas present up to 50% of inhabitants exposed to the fungus, but only a minority develops the disease [4]. In Brazil, the mortality rate of PCM for 1.000.000 of inhabitants was 1.45 between 1980-1995, and 0.9-1.0 between 1996-2006. According to this mortality rate, the number of annual new cases in Brazil is about 3360 [6].

PCM presents two forms of disease: the acute/juvenile and the chronic/adult forms. The first one affects children and adolescents of both genders, and represents 5-15% of all cases. This form is characterized by an aggressive evolution, frequently with skin lesions, digestive symptoms and lymphadenopathy. The chronic form is more common in male adults, and has a slowly progression with pulmonary symptoms present in more than 90% of patients [4,7,8,9].

The evolution of PCM is associated to many factors as host immune response and fungus virulence. The cellular immune response is known as essential for host defense against the fungus [10]. Mild and chronic forms of PCM are related to the production of low levels of antibodies whereas patients with severe and acute PCM present high levels of antibodies [11]. It is suggested that the Th1 pattern of immunological response is associated with asymptomatic and mild forms of PCM, and the Th2 pattern would be related to severe disease [12-18]. Patients with acute disease generally present high levels of type 2 cytokines such as IL-4, IL-5, and TGF-β [19]. Patients with chronic disease seem to have an intermediate pattern of immune response with Th1 (IFN-γ, IL-12, IL-2) and Th2 cytokines [10]. Some factors may influence the development of a Th1 or a Th2 pattern of immune response as host and pathogen genetic background, fungal load and virulence, but there is not a clear consensus about this [20, 21].

Different drugs are used to treat patients with PCM, as sulfonamides, amphotericin B and imidazole derivatives. The drug selection is generally based on disease severity, but the treatment cost is a relevant factor in the drug choice [8]. The determination of the exact time to discontinue therapy of patients with PCM remains an important issue. The serological cure criteria available and applied in patients treated for PCM are not reliable in some cases. Many patients considered clinically cured, present high levels of antibodies even after...
years of treatment interruption. Actually, there is not a reliable laboratory parameter that is associated with disease activity in patients with PCM that allows the conclusion that they are cured.

Serological techniques are largely used in the diagnosis of PCM, but one problem with tests applying crude antigens is the cross reaction with other fungi. Many different antigens have been tested in the diagnosis of PCM, but there is not a molecule that can be applied as a gold standard. To improve serological tests, different antigens are proposed as more specific to diagnose patients with PCM. Proteins of surface and cytosol, like Pb27, have been described and characterized as suitable antigens for serological diagnosis of PCM [22,23]. The mexo antigen, a secreted and surface protein, have demonstrated excellent results in the diagnosis of PCM, and it has been indicated as a marker of active disease [24].

Disease activity of many disorders as arthritis, tuberculosis, leprosy, malaria, typhoid fever and endocarditis has been associated with high serum concentrations of soluble TNF-α receptors (sTNF-R) [25-30]. Some chemokines also present an association between their high concentrations and active disease [30-33]. In PCM, the relation between high concentrations of sTNF-R, chemokines and active disease has been proposed by different authors [32-35].

The sTNF-R1, sTNF-RII and the chemokine CXCL9 demonstrated value in monitoring patients during treatment. These serological markers presented decreased concentrations simultaneously to clinical remission of symptoms of PCM, showing their potential application as serological markers in the control of cure of this disease [33].

This study aimed to measure the levels of IgG anti- P. brasiliensis and the concentrations of sTNF-R1, sTNF-RII, CCL2, CCL3, CXCL9, CCL11 and CCL24 in the sera of patients treated for PCM to verify the applicability of these serological markers in the control of cure of this disease.

Patients and Methods

Patients and sera

Sera were collected from patients with acute and chronic PCM at the Centro de Treinamento e Referência em Doenças Infecciosas e Parasitárias (CTF-DIP) of the General Hospital of the Universidade Federal de Minas Gerais (UFMG), Brazil. Determination of serological markers was performed in 19 patients during treatment (dt); in 22 patients at the day of treatment interruption (t0); in 22 patients at six months after treatment interruption (t6); in 18 patients at 12 months (t12); in 13 patients at 18 months (t18); in nine patients at 24 months (t24); in six patients at 30 months (t30); in five patients at 36 months (t36) and in three patients at 42 months after treatment interruption (t42). Sera were frozen and stored at -20°C until use. The diagnosis of PCM was made by biopsy in all patients and in some cases conventional serological tests were used combined with biopsy.

Patients were treated with ketoconazole (3), itraconazole (1), sulfamethoxazole-trimethoprim (21) or sulfamethoxazole-trimethoprim associated with amphotericin B (1) during hospitalization. The patients analyzed were not treated with immunosuppressive drugs.

The group analyzed was formed by 23 patients with chronic PCM (4 women and 19 men) and three patients with acute PCM (two women and one man), with 49.5 ± 16.5 (mean ± standard deviation) years old. Ten health volunteers with similar parameters of age (44.9 ± 13.3) and sex (three women and seven men) formed a negative control (NC) group. This study was approved (protocol n° 00904104100-09) by the ethic committee of the UFMG and informed consent was obtained from each patient before blood collection. Patients with other concomitant diseases as toxoplasmosis, histoplasmosis, cryptococcosis, infectious mononucleosis, immune deficiency syndrome (AIDS), tuberculosis, sarcoidosis and lymphoma were excluded from the study.

Antigens used in the ELISA

The Mexo antigen was obtained from yeast cells cultured in YPD agar (0.5% yeast extract, 0.5% peptone, 1.5% D-glucose, 1.5% agar, pH 7.0) medium (Sigma, St. Louis, MO, USA) a 35°C. In the 7th day of culture, the yeast cells were removed from the culture medium and submitted to agitation in a vortex in 0.05 M PBS, pH 7.4, for 30 s. The protein content of the supernatant was determined and it was used as antigen.

The production of Pb27 antigen involves the culture of yeast cells as mentioned above, and a process of fractionation and filtration chromatography. The fractions obtained were sequenced, cloned and expressed to produce a purified recombinant Pb27 protein as described by [23].

Measurement of serological markers

The measurement of all serological markers tested was made in duplicate, and if any discordant result was found, another dosage was proceeded.

ELISA for measure levels of total IgG anti- P. brasiliensis

The ELISA for measure levels of total IgG anti- P. brasiliensis was performed in flat-bottomed polystyrene plates (Nunc-ImmunoPlate Polysorp Surface, USA) using Mexo and recombinant Pb27 (rPb27) as antigens. Briefly, plates were coated overnight at 4°C with 100 μL of a 1 μg/100 μL solution of Mexo or rPb27 in a 0.5 mol L\(^{-1}\) carbonate-bicarbonate buffer, pH 9.6. The plates were washed five times with a washing solution and blocked with 200 μL of blocking solution for 1 h at 37°C. After incubation, plates were washed and filled with 100 μL of either patient sera or negative control sera (in duplicate) diluted 1:400. The plates were re-incubated for 1 h at 37°C and then washed. After washing, 100 μL of a peroxidase conjugate anti-human IgG specific to the gamma chain (DAKO, USA) diluted 1:10,000 were added to the wells. The plates were incubated for 1 h at 37°C and then washed. The reaction was developed with 100 μL of TMB Plus (Bio-technologia, Brazil) for 10 min at room temperature. Colour development was stopped with 2 mol L\(^{-1}\) H\(_2\)SO\(_4\). The optical density (OD) at 450 nm was determined using an ELISA reader (Anthos 2010, Cambridge, England). Cut-off values were determined by the construction of the Receiver Operator Characteristic (ROC) curve with ten sera samples from NC group.

ELISA for measure concentrations of soluble TNF-α receptors and chemokines

Concentrations of sTNF-R1, sTNF-RII, CCL2, CCL3, CXCL9, CCL11 and CCL24 were measured with a capture ELISA technique using kits DuoSet® ELISA Development System (R&D Systems, USA). The technique was performed according to the manufacturer protocol. The concentrations of the serological markers were determined based on a standard curve for each set of samples analyzed. Cut-off values
were determined by the construction of the ROC curve with ten sera samples from NC group.

**Statistical analysis**

All data were plotted in dispersion graphs in which lines represent median. Serological results of PCM patients versus NC group were analyzed by Mann-Whitney test. All groups were compared and analyzed by Kruskall-Wallis non-parametric test and Dunn’s post-test. Spearman test was performed to assess correlation between the studied parameters. All calculations were performed using GraphPad Prism version 4.00 for Windows software (GraphPad Prism, CA, USA). ROC curves were constructed using MedCalc statistical program (Broekstraat, Mariakerke, Belgium) to define the cut-off points of the serological markers analyzed. The chosen cut-off value for each marker was the one that maximized the sum of sensitivity and specificity. Data were considered significant when p<0.05.

**Results**

The 23 patients analyzed with chronic PCM presented predominantly mucosal, skin, lung and brain manifestations when the disease was active. The three patients with acute disease presented lymphadenomegaly as the major manifestation (Table 1). According to the clinical analysis performed every six months, none of the patients presented relapse of PCM during all the period of 42 months of follow-up after the interruption of treatment. It is important to mention that even patients that were excluded from the study were evaluated in aleatory moments and they were clinically cured.

<table>
<thead>
<tr>
<th>Clinical Form</th>
<th>Lesions</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute</td>
<td>Lymph nodes</td>
<td>3 (11.54)</td>
</tr>
<tr>
<td>Chronic Unifocal Tegumentary</td>
<td>Lymph nodes</td>
<td>2 (7.69)</td>
</tr>
<tr>
<td></td>
<td>Oral mucous</td>
<td>4 (15.38)</td>
</tr>
<tr>
<td></td>
<td>Larynx</td>
<td>4 (15.38)</td>
</tr>
<tr>
<td></td>
<td>Skin</td>
<td>1 (3.85)</td>
</tr>
<tr>
<td>Chronic Unifocal Pulmonary</td>
<td>Lungs</td>
<td>1 (3.85)</td>
</tr>
<tr>
<td>Chronic Multifocal</td>
<td>Skin, oral mucous</td>
<td>4 (15.38)</td>
</tr>
<tr>
<td></td>
<td>Skin, lungs</td>
<td>1 (3.85)</td>
</tr>
<tr>
<td></td>
<td>Skin, brain, lungs</td>
<td>1 (3.85)</td>
</tr>
<tr>
<td></td>
<td>Skin, oral mucous, lymph nodes</td>
<td>1 (3.85)</td>
</tr>
<tr>
<td></td>
<td>Oral mucous, lymph nodes</td>
<td>3 (11.53)</td>
</tr>
<tr>
<td></td>
<td>Lungs, brain</td>
<td>1 (3.85)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>26 (100)</td>
</tr>
</tbody>
</table>

Table 1: Clinical forms of patients treated for paracoccidioidomycosis and considered cured.

<table>
<thead>
<tr>
<th>Serum marker</th>
<th>AUC</th>
<th>p value</th>
<th>Cut-off (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total IgG using Mexo</td>
<td>0.95</td>
<td>&lt;0.0001*</td>
<td>0.126#</td>
</tr>
<tr>
<td>Total IgG using rPb27</td>
<td>0.938</td>
<td>&lt;0.0001*</td>
<td>0.136#</td>
</tr>
<tr>
<td>sTNF-RI</td>
<td>0.995</td>
<td>&lt;0.0001*</td>
<td>1192.308</td>
</tr>
<tr>
<td>sTNF-RII</td>
<td>1</td>
<td>&lt;0.0001*</td>
<td>3661.428</td>
</tr>
<tr>
<td>CXCL9</td>
<td>0.487</td>
<td>0.8614</td>
<td>250</td>
</tr>
<tr>
<td>CCL2</td>
<td>0.974</td>
<td>&lt;0.0001*</td>
<td>42.138</td>
</tr>
<tr>
<td>CCL3</td>
<td>0.64</td>
<td>0.1434</td>
<td>650</td>
</tr>
<tr>
<td>CCL11</td>
<td>0.561</td>
<td>0.528</td>
<td>290</td>
</tr>
<tr>
<td>CCL24</td>
<td>0.775</td>
<td>0.0040*</td>
<td>2452.72</td>
</tr>
</tbody>
</table>

*Statistical significance p<0.05

Table 2: Area under the ROC curve (AUC), p value and cut-off values of serological markers analyzed in patients treated for paracoccidioidomycosis and considered cured.

The values of ROC curve statistics are listed in Table 2. The p value <0.0001 and the high values of area under the curve (AUC) for total IgG using Mexo and rPb27 as antigens demonstrated that this serological marker segregated the majority of patients treated for PCM from the NC group. During the treatment for PCM, the majority of patients presented high levels of total IgG using Mexo as antigen, as it was expected. However, two patients were not reactive to Mexo when the disease was active. These two patients presented levels of total IgG below the cut-off point during the treatment and after its interruption during all the period analyzed, and they were never reactive to the antigen. The other patients presented high levels of IgG even after 42 months of interruption of treatment without a progressive decrease in these values over the period after its interruption. It was possible to verify a decrease in the levels of IgG for some patients, but generally they remained with similar values over the period analyzed. The measure of total IgG using rPb27 as antigen presented similar results. Probably, more patients presented less reactivity with this antigen due to the high specificity of rPb27, which was not recognized by some patients. The serological levels of total IgG with both antigens were similar for all patients at the different periods analyzed. The analysis of total IgG using both antigens demonstrated statistical difference between dt, t0, t6, t12, t18 and t30 groups from NC group. There was not statistical difference among the groups of patients treated for PCM (Figure 1). It was not found statistical correlation between IgG levels using Mexo or rPb27 as antigens and the period analyzed.
Figure 1: Serum levels of total IgG measure by an in house ELISA using Mexo and rPb27 as antigens in the sera of 19 patients with PCM during the treatment (dt); in 22 patients at the time of interruption of treatment (t0); in 22 patients at six months (t6); in 18 patients at 12 months (t12); in 13 patients at 18 months (t18); in nine patients at 24 months (t24); in six patients at 30 months (t30); in five patients at 36 months (t36) and in three patients at 42 months after interruption of treatment (t42) and in 10 healthy individuals (NC). Each dot represents the optical density of a single patient and the horizontal line the median of the group. The cut-off point is represented by the dotted line. Data marked by ‘a’ were significantly different (p<0.05) from NC group.

The high values of AUC and the p<0.0001 presented by sTNF-RI, showed that this marker discriminated practically all patients treated for PCM from NC group (Table 2). The sera concentrations of sTNF-RI were high in all patients with PCM, with the exception of only one patient, that presented the concentration of sTNF-RI during the treatment below the cut-off point. This patient presented an increase in the concentration of sTNF-RI up to the moment of interruption of treatment, which was maintained over the period analyzed. It was observed that patients during the treatment presented concentrations of sTNF-RI slightly elevated when compared to other groups. Statistical difference was observed between dt, t0, t6, t12, t18, t24, t36 and NC group. Among the groups of patients with PCM, only the groups dt and t6 presented statistical difference (Figure 2). It was not found statistical correlation between the concentrations of sTNF-RI and the period analyzed.

Figure 2: Serum concentrations of sTNF-RI and sTNF-RII measure by ELISA in the sera of 19 patients with PCM during the treatment (dt); in 22 patients at the time of interruption of treatment (t0); in 22 patients at six months (t6); in 18 patients at 12 months (t12); in 13 patients at 18 months (t18); in nine patients at 24 months (t24); in six patients at 30 months (t30); in five patients at 36 months (t36) and in three patients at 42 months after interruption of treatment (t42) and in 10 healthy individuals (NC). Each dot represents the concentration of a single patient and the horizontal line the median of the group. The cut-off point is represented by the dotted line. Data marked by ‘a’ were significantly different (p<0.05) from NC group; data marked by ‘b’ were significantly different (p<0.05) from dt group.

sTNF-RII presented the best results of ROC curve analysis, with maximum of AUC, what allowed this serological marker to segregate all patients treated for PCM from NC group (Table 2). Patients during the treatment presented higher concentrations when compared to other groups. It was observed statistical difference between dt, t0, t6, t12, t24, t36 and NC group. Among the groups of patients with PCM it
was verified statistical difference between t0, t6, t12, t18 and dt group (Figure 2). It was found negative correlation between the concentrations of sTNF-RII and the period analyzed.

The majority of patients presented concentrations of CXCL9 below the cut-off point during the treatment and at all periods after its interruption. Generally, patients remained with similar concentrations of CXCL9 over the period analyzed, without increase or decrease in the values over the time. Only one patient presented increased concentrations of CXCL9 at the day of interruption of treatment, at 12 and 18 months after the interruption of treatment. It was observed statistical difference between dt and NC group. Among the patients with PCM it was found statistical difference between t0, t12, t24, t30 and dt groups (Figure 3). It was found positive correlation between the concentrations of CXCL9 and the period analyzed.

For CCL2 it was observed that the majority of patients during the treatment presented concentrations of this chemokine above the cut-off point, as it was found at the moment of its interruption. Up to six months after interruption of treatment, it was verified that concentrations of CCL2 decreased progressively over the time. Among the patients with PCM it was found statistical difference between dt, t0, t12 and t18 and NC group (Figure 3). It was found negative correlation between the concentrations of CCL2 and the period analyzed.

In the analysis of CCL3, it was verified that the majority of patients presented concentrations of this marker above the cut-off point during the treatment and at the moment of its interruption. These concentrations presented a tendency to decrease over the time, with only two patients (2/9) presenting detectable concentrations of CCL3 at 24 months after interruption of treatment; two (2/6) patients at 30 months and one (1/5) patient at 36 months after interruption of treatment. It was not found statistical difference between the groups analyzed (Figure 3). Negative correlation was found between the concentrations of CCL3 and the period analyzed.

The analysis of CCL11 demonstrated that practically all patients presented concentrations below the cut-off point during the treatment. Only one patient presented the concentration of CCL11 above the cut-off point during the treatment and at the moment of its interruption. These concentrations presented a tendency to decrease over the time, with only two patients (2/9) presenting detectable concentrations of CCL11 at 24 months after interruption of treatment; two (2/6) patients at 30 months and one (1/5) patient at 36 months after interruption of treatment. It was not found statistical difference between the groups analyzed (Figure 3). Negative correlation was found between the concentrations of CCL11 and the period analyzed.

Figure 3: Serum concentrations of CXCL9, CCL3, CCL11 and CCL24 measure by ELISA in the sera of 19 patients with PCM during the treatment (dt); in 22 patients at the time of interruption of treatment (t0); in 22 patients at six months (t6); in 18 patients at 12 months (t12); in 13 patients at 18 months (t18); in nine patients at 24 months (t24); in six patients at 30 months (t30); in five patients at 36 months (t36) and in three patients at 42 months after interruption of treatment (t42) and in 10 healthy individuals (NC). Each dot represents the concentration of a single patient and the horizontal line the median of the group. The cut-off point is represented by the dotted line. Data marked by 'a' were significantly different (p<0.05) from NC group; data marked by 'b' were significantly different (p<0.05) from dt group.
off point during the treatment, however, after its interruption, the concentrations presented by this patient decreased and remained below the cut-off point. From the moment of interruption of treatment, the concentrations of CCL11 were constant over the time. Patients that presented increase in these values up to the interruption of treatment maintained high concentrations during all the time. On the contrary, some patients remained with concentrations of CCL11 below the cut-off point during all the time. It was observed statistical difference between t0, t12 and dt groups (Figure 3). It was not found statistical correlation between the concentrations of CCL11 and the period analyzed.

Although CCL24 presented p=0.004, it was not possible to discriminate the majority of patients treated for PCM from the NC group (Table 2). It was not found a pattern of increase or decrease of concentrations during and after treatment. Generally, patients remained with constant concentrations of CCL24 during all the period. It was not found any difference between the groups analyzed (Figure 3). It was not observed statistical correlation between the concentrations of CCL24 and the period analyzed.

It was not found any tendency in the levels of serological markers tested or in clinical manifestations presented by patients, related to sex and age.

Discussion

The serological levels of IgG remained high in the majority of patients analyzed over the time independently of the antigen used. It was possible to distinguish patients with PCM from NC group, even at 42 months after treatment interruption. It is worth to mention that all patients analyzed were considered cured based on clinical criteria of the Guidelines on PCM [8]. It is proposed by these Guidelines a serological criterion based on negative conversion or stabilization of serological titers using double immunodiffusion, in two sera samples every six months after the recommended period of treatment. In our study very few patients would be considered cured based on the serological criteria of these Guidelines, despite all of them present clinical cure. A similar result was also verified in other study made by our group with different patients before, during and after treatment. High levels of IgG using Mexo and rPb27 as antigens were also detected even three years after treatment, without a clear association between progression of treatment and decrease in IgG levels [36]. Since the levels of IgG may persist high for long periods, even when the disease is inactive, these studies show that this marker may not be the safest for monitoring patients during and after treatment for PCM.

Generally, antibody serological titers are elevated in patients with acute or severe chronic PCM, while patients with unifocal chronic PCM may present low antibody titers [3,8,37]. Unlike other reports, in this work, patients with unifocal and mild disease presented similar levels of IgG when compared to patients with multifocal and severe PCM. Two patients with multifocal disease presented levels of IgG below the cut-off point even when PCM was active. The three patients with acute PCM presented reactivity with the antigens used, but the levels of IgG were lower when compared to patients with chronic PCM. This difference found between our work and other studies could be explained by many factors such as the genetic, geographical and immunological profile of the population analyzed, the genetic of the fungus and the serological technique applying the different antigens Mexo and rPb27. This result suggests that the clinical manifestations of PCM have an important and not well known relationship to the immunological response.

The sera concentrations of sTNF-RI and sTNF-RII remained high even at 42 months after interruption of treatment in the patients analyzed. It was verified a decrease in the values of both chemokines after the interruption of treatment, compared to the period during treatment. However, it was possible to segregate the majority of patients with PCM from the NC group during all the period of the study. Only one patient with unifocal chronic PCM presented concentrations of sTNF-RI below the cut-off point during the treatment. Our results are discordant from those of Corvino et al. [32] who detected higher serological concentrations of sTNF-RII in patients with acute PCM, with a marked and progressive decrease over two years of treatment. Patients with chronic PCM presented higher concentrations of sTNF-RI, with a gradual decrease over the period of treatment. It was suggested that sTNF-RII was closely related to the clinical course of PCM, and could be used as a severity marker. Concentrations of sTNF-RI were not strongly affected by the treatment and any association to the clinical course of PCM was found. Although, similar to our findings, Corvino et al. [32] demonstrated that in patients with chronic PCM the serological concentrations of sTNF-RII remained constant over the period of treatment, and patients with acute PCM presented increase in serological concentrations of sTNF-RI over the period of treatment. Moura et al. [33] detected high concentrations of sTNF-RI and sTNF-RII in patients with active chronic PCM before and during treatment. It was observed that the decrease of the serological concentrations of sTNF-RI and sTNF-RII over the period of treatment was associated with the extinction of symptoms of PCM. They suggested a relation between high serological concentrations of sTNF-RI and sTNF-RII and active PCM, with the use of these markers to monitoring patients during treatment. However, according to the conclusion of Moura et al. [33], the patients analyzed in our study should present clinical manifestations of PCM, since all of them presented high concentrations of sTNF-RI and sTNF-RII even after 42 months of treatment interruption. These two serological markers are related to inflammatory response, especially during active disease. Although, the exact role of these two receptors in PCM is not explained. One explanation for the high concentrations of sTNF-RI and sTNF-RII detected in our patients could be a residual or persistent activation of the immunological system by P. brasiliensis that could be in a latent form. In this way, even after years of interruption of treatment, and without clinical manifestations, patients would still present detectable concentrations of sTNF-RI and sTNF-RII. This result indicates these markers are not safe to be applied in monitoring patients after interruption of treatment for PCM.

The majority of patients presented low concentrations of CXCL9 during the 42 months of follow-up. Generally, the patients presented similar concentrations of CXCL9 over the time, without significant increase or decrease. Only one patient presented an increase in concentrations, reaching the maximum at 18 months after treatment interruption. Moura et al. [33] analyzed patients with chronic PCM before and during treatment, and it was verified that concentrations of CXCL9 decreased over the period of 36 months of treatment. It was proposed that high concentrations of this chemokine would be associated with active PCM, since the absence of symptoms was directly related to low concentrations of CXCL9. The high concentrations of this chemokine during the active disease could be explained by the need to attack the fungus, since CXCL9 is related to Th1 immune response. CXCL9 can attract Th1 cells and block Th2 cells from acting against the fungus.
cells and this function is necessary in patients with active disease, when *P. brasiliensis* must be destroyed [38]. Corvino et al. [32] also verified high concentrations of CXCL9 in patients with PCM without treatment, and an association between active disease and higher concentrations of CXCL9 was found. Souto et al. [39] demonstrated that IFN-γ modulates the production of chemokines in the lungs of mice infected with *P. brasiliensis*, and induces the expression of Th1 chemokines. In our study all patients after treatment were considered clinically cured, so it was expected that they would present low concentrations of CXCL9. The high concentration found in only one patient after the interruption of treatment could not be explained, since he did not present any clinical manifestation of PCM during the increase of CXCL9 concentration. The low concentrations of CXCL9 during the treatment, when patients presented active disease, were not expected. In the study of Moura et al. [33] six from 26 patients did not presented detectable CXCL9 before treatment. In our study, it was also expected that more patients would present high concentrations of CXCL9 during treatment. One possibility for the low concentrations found in the patients during treatment could be an inefficient and variable production of CXCL9 by these patients. Since other inflammatory markers as sTNF-RI and sTNF-RII related to Th1 response, and CCL11 and CCL3 related to Th2 response were detected at high concentrations in the same patients, the absence of a protective and established immunological response could not be the explanation for the low values of CXCL9 during treatment. It could be an isolate fact related to CXCL9, although it is not possible to better explain it.

It was possible to verify that during the treatment the majority of patients presented high concentrations of CCL2. Up to treatment interruption, many patients presented a progressive decrease in the concentrations of CCL2, however, even after 42 months of follow-up, some patients still presented detectable CCL2, when compared to NC group. Moura et al. [33] verified that patients with chronic PCM presented an increase in the concentrations of CCL2 during the first year of treatment. Our results suggest that after treatment interruption, the concentrations of CCL2 present a tendency to decrease. However, as it was not possible to find basal values of this chemokine in all patients and the decrease in these values were slow, CCL2 was not considered safe for monitoring patients in control of cure of PCM.

In our study, the concentrations of CCL3 during the treatment did not present relevant difference compared to the other periods analyzed. However, the concentrations of CCL3 appear to decrease after the interruption of treatment, what makes sense. This chemokine acts in the recruitment of monocytes and T cells, consequently, it is expected that in patients considered cured the concentrations of CCL3 should be low, since the presence of protective cells attacking the fungus is no longer necessary. Moura et al. [33] verified increased concentrations of CCL3 over the period of 12 months of treatment in patients with chronic PCM. This result was explained as a transitory elevation of Th2 pattern of immune response during treatment of patients when the fungal burden was controlled and it was not necessary a strong Th1 response. Although it was found a negative correlation between the concentrations of CCL3 and the period after interruption of treatment, this chemokine could not be used safely to monitoring patients in control of cure since these concentrations did not reach basal values for all patients.

During the treatment, the majority of patients presented concentrations of CCL11 below the cut-off point. After the treatment, many patients presented high concentrations of this chemokine, without statistical difference between the groups analyzed. These high concentrations of CCL11 could not be explained, since this chemokine is related to attraction to protective cells in the establishment of immune response, and the patients analyzed did not present any symptom of PCM. Maybe the high concentrations found could be a residual immune response initiated during the treatment that persisted longer. A similar result was found for CCL24, although, for this chemokine the concentrations were higher during the treatment. It was related by Moura et al. [33] that CCL24 would have their concentrations increased at 36 months of treatment, probably to balance the strong Th1 response established during the active disease. These results show the impossibility to use these markers in the control of cure of PCM.

It was verified that none of the serological markers tested presented concentrations at basal line for all patients. The high levels detected for total IgG indicate that the serological criteria based on negative conversion or stabilization of IgG levels should not be applied for all patients. The same situation was found for sTNF-RI and sTNF-RII, what shows that the clinical criterion for interruption of treatment may not be reliable. One explanation for the high values of the majority of markers analyzed maybe a persistent inflammation presented by the patients, even if they do not have symptoms of PCM. Although CXCL9 presented an association between low concentrations and absence of symptoms of PCM after the interruption of treatment, due to its low values detected during treatment, it was not possible to indicate this marker for monitoring patients in control of cure.

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**Conflict of Interest**

The authors declare that there is no conflict of interest.

**References**