FACULDADE DE ODONTOLOGIA

INTER-RELAÇÃO DE ASPECTOS CLÍNICOS, HISTOMORFOMÉTRICOS E IMUNOISTOQUÍMICOS NA PARACOCCIDIOIDOMICOSE ORAL

MARIANA ÀLVARES DE ABREU E SILVA

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INTERRELATIONSHIP OF CLINICAL, HISTOMORPHOMETRIC AND IMMUNOHISTOCHEMICAL FEATURES IN ORAL PARACOCCIDIOIDOMYCOSIS

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Há homens que lutam um dia, e são bons;
Há outros que lutam um ano, e são melhores;
Há aqueles que lutam muitos anos, e são muito bons;
Porém há os que lutam toda a vida;
Estes são os imprescindíveis."

Bertold Brecht (1898-1956)

Dedicatória

Dedico este trabalho aos meus maiores incentivadores:

Meus pais, Antônio José e Angélica Maria, minha irmã Fabiana

e meu sobrinho Leonardo.

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Resumo

RESUMO

O presente estudo teve por objetivo analisar características histomorfométricas, imunoistoquímicas e clínicas de lesões orais da paracoccidioidomicose. A amostra foi composta por 50 prontuários e 50 blocos de parafina contendo espécimes biopsiados de lesões orais, ambos provenientes de pacientes portadores de paracoccidioidomicose diagnosticados no Serviço de Estomatologia do Hospital São Lucas da PUCRS no período compreendido entre os anos de 1977 e 2010. Informações sobre tempo de evolução da doença, número e tamanho das lesões orais, bem como contagem de eritrócitos, leucócitos, linfócitos, hematócrito, hemoglobina e eritrossedimentação foram coletadas dos prontuários dos pacientes. Cortes histológicos obtidos a partir dos espécimes em parafina foram submetidos às colorações de hematoxilina e eosina (H&E), Gomori-Grocott e processamento imunoistoquímico. As amostras foram agrupadas de acordo com a intensidade de compactação do granuloma, e as variáveis número de fungos, número de brotamentos, diâmetro dos fungos, diâmetro dos brotamentos, expressão imunoistoquímica de IL-2, TNF-alfa e IFN-gama foram avaliadas e correlacionadas. O diâmetro dos brotamentos foi significativamente maior nos granulomas de compactação intermediária quando comparados aos granulomas de maior compactação. As demais variáveis (número de brotamentos, número e diâmetro dos fungos, expressão de IL-2, TNF-alfa e IFN-gama, características clínicas e hematológicas) não exibiram alteração significativa de acordo com o grau de compactação dos granulomas. Foi observada correlação positiva entre número de brotamentos e número de fungos (r=0.834); diâmetro dos brotamentos e diâmetro do fungo (r=0.496); eritrócitos e número de fungos (r=0.420); eritrócitos e número de brotamentos (r=0.408); leucócitos e número de brotamentos (r=0.396). A correlação negativa ocorreu entre diâmetro e número de fungos (r=-0.419); diâmetro dos brotamentos e compactação do granuloma (r=-0.367); expressão de TNF-alfa e número de fungos (r=-0.372); expressão de TNF-alfa e número de brotamentos (r=-0.300). As características histológicas, imunológicas e clínicas das lesões orais da paracoccidioidomicose crônica não diferiram significativamente entre os pacientes da amostra avaliada. Os níveis de TNF-alfa nas lesões orais estão inversamente relacionados à intensidade da infecção.

Palavras-chave: citocinas, imunoistoquímica, paracoccidioidomicose, Fator de necrose tumoral-alfa, Interferon-gama, Interleucina-2, *Paracoccidioides brasiliensis*.

Summary

SUMMARY

The present study aimed at analyzing histomorphometric, immunohistochemical and clinical features of oral lesions of paracoccidioidomycosis. The sample comprised 50 medical charts and 50 paraffinized blocks of biopsed specimens of oral leisons, both from paracoccidioidomycosis patients diagnosed at Stomatology Department of Hospital São Lucas, PUCRS from 1977 to 2010. Data regarding disease duration, and size and number of oral lesions, as well as erythrocytes, leukocytes, lymphocytes, hematocrit, hemoglobin and erythrocyte sedimentation rate, were collected from medical charts. Histological cuts were obtained from the paraffinized specimens and subjected to hematoxylin and eosin (H&E), Gomori-Grocott and immunohistochemical staining. The sample was classified according to the density of granulomas, and the variables number and diameter of fungi, number and diameter of buds, and IL-2, TNF-alpha and IFN-gamma expression were analyzed and correlated. Bud diameter was significantly greater in intermediate density granulomas compared to higher density granulomas. The other variables (bud number, number and diameter of fungi, expression of IL-2, TNF-alpha and IFN-gamma, and clinical and hematological features) did not significantly change with the density of granulomas. There was a positive correlation between bud number and fungal cell number (r=0.834), bud diameter and fungal cell diameter (r=0.496), erythrocytes and number of fungi (r=0.420), erythrocytes and bud number (r=0.408), and leukocytes and bud number (r=0.396). Negative correlation occurred between number and diameter of fungal cells (r=-0.419), bud diameter and granuloma density (r=-0.367), TNF-alpha expression and number of fungal cells (r=-0.372), TNF-alpha expression and bud number (r=-0.300). Histological, immunological and clinical characteristics of oral lesions of chronic paracoccidioidomycosis did not differ significantly between patients in our sample. TNF-alpha levels in oral lesions were inversely correlated with intensity of infection.

Keywords: Cytokines, Immunohistochemistry, Paracoccidioidomycosis, Interleukin-2, Tumor necrosis factor-alpha, Interferon-gamma, *Paracoccidioides brasiliensis*.

Sumário

SUMÁRIO

Introdução

1 INTRODUÇÃO

A paracoccidioidomicose é uma micose sistêmica, endêmica da América Latina (Shikanai-Yasuda *et al*., 2006), que tem o Brasil como principal representante (Santo, 2008). Nos estados de São Paulo, Rio de Janeiro, Minas Gerais, Paraná, Rio Grande do Sul, Goiás e Mato Grosso do Sul constitui problema de saúde pública pelas despesas com os casos da doença ativa e pelas sequelas, que podem impedir o retorno ao trabalho (Bava *et al*., 1991; Kashino *et al*., 2000; Lyon *et al*., 2009; Pedroso *et al*., 2009).

O agente etiológico da doença é o *Paracoccidioides brasiliensis*, fungo dimórfico que se encontra na natureza sob a forma de micélio e, após ser inalado, transforma-se na forma patogênica de levedura (Shikanai-Yasuda *et al*., 2006). Os pulmões são o primeiro sítio atingido e, por disseminação sanguínea ou linfática do fungo, outros sítios podem ser acometidos, entre eles a cavidade oral (Lyon *et al.*, 2009; Neworal *et al*., 2003; Souto *et al*., 2000).

Apesar de a maioria da população das áreas endêmicas estar infectada, apenas uma minoria imunologicamente incompetente manifestará a doença (Ramos-e-Silva; Saraiva, 2008). O grau de comprometimento da resposta imunológica celular determinará a gravidade e a forma clínica da doença (Bava *et al*., 1991; Fornari *et al*., 2001), que pode assumir duas apresentações: aguda/subaguda e crônica. A forma aguda afeta crianças e adolescentes de ambos os sexos de maneira disseminada e agressiva, enquanto a forma crônica, mais localizada, atinge homens acima dos 30 anos de idade, geralmente tabagistas e etilistas (Martins *et al*., 2003; Shikanai-Yasuda *et al*., 2006). As lesões orais têm aspecto moriforme, sendo geralmente multicêntricas e dolorosas, associadas a macroqueilia, sialorreia e linfadenopatia cervical (Martins *et al*., 2003). Ao exame histológico, observa-se

o granuloma epitelioide, uma espécie de resposta imunológica específica contra o fungo e sua disseminação pelo organismo (Martinez *et al*., 1996).

A resposta imunológica celular do tipo Th2 com alta produção de IL4, IL5, IL10 e anticorpos, ativação policlonal de células B e comprometimento da produção de IFN-gama (Kashino *et al*., 2000; Livonesi *et al*., 2009) está associada à formação do granuloma frouxo (Almeida *et al*., 2003), que é característico da forma disseminada aguda da doença. Já o granuloma compacto é típico da forma localizada (Iabuki; Montenegro 1979; Martinez *et al*., 1996) ou benigna, em que há predomínio da resposta imunológica celular do tipo Th1 com produção de IFN-gama, IL2 e TNF-alfa e baixos níveis de IL4, IL5, IL10 e anticorpos (Livonesi *et al*., 2009; Kashino *et al*., 2000). Assim, o desequilíbrio na produção de citocinas pró e anti-inflamatórias contribui para o estabelecimento da doença (Benard *et al*., 2001; Fornari *et al*., 2001).

Embora as características microscópicas da paracoccidioidomicose sejam bem conhecidas, a literatura prescinde de estudos que explorem os aspectos histológicos das lesões orais da doença comparando-os com a situação clínica e imunológica dos pacientes. O presente estudo teve por objetivo classificar o padrão histológico das lesões orais de paracoccidioidomicose por meio de histomorfometria em hematoxilina e eosina (H&E) e relacioná-lo com a quantificação do fungo e brotamentos, expressão imunoistoquímica de TNF-alfa, IFN-gama e IL-2, bem como com aspectos clínicos da doença.

2 ARTIGO 1

O artigo a seguir intitula-se **Important aspects of oral paracoccidioidomycosis – a literature review** e foi formatado de acordo com as normas do periódico *Mycoses* (Anexos A e B).

Important aspects of oral paracoccidioidomycosis – a literature review

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Running head: *Oral paracoccidioidomycosis*

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Summary

Paracoccidioidomycosis is a deep mycosis endemic to Latin America, with considerable morbidity and mortality. It is caused by the dimorphic fungus *Paracoccidioides brasiliensis*, which affects, among other organs in the human body, the oral cavity. Fungus virulence and immunocompetence of the host determine the establishment of infection or active disease, whose severity and clinical behavior depend mostly on the cellular immune response of the host. Often, oral lesions constitute the first sign and site of confirmation of diagnosis, which in most cases is delayed. The success of the treatment depends on early and correct diagnosis, as well as on the patient's adherence to the drug therapy.

Introduction

Paracoccidioidomycosis, also known as Lutz disease or Lutz-Splendore-Almeida mycosis, is a deep systemic disease, considered the principal endemic mycosis in Latin America [1-4]. It was first described by Adolpho Lutz in 1908 who examined a patient with oral lesions and cervical lymphadenopathy, from which the fungus was isolated. The morphological and biological characterization of the pathogen was made by Alfonso Splendore in 1912, who called it *Zymonema brasiliensis*, and by Floriano Paulo Almeida, who proposed the name *Paracoccidioides brasiliensis* in 1930. The name South American blastomycosis was used for a long time, but it was withdrawn because the disease also occurs in Central America and Mexico. In 1971, the name paracoccidioidomycosis was definitely adopted [5-8]. Although the autochthonous cases are restricted to the endemic zone, the disease can occur beyond these geographical limits, and it is important to consider the possibility of very long periods of latency of the fungus in the human host. We present here a literature review focusing on important clinical, histological and immunological aspects of oral paracoccidioidomycosis.

Etiopathogenesis

Paracoccidioides brasiliensis is a dimorphic fungus found in nature between latitudes 23ºN and 34ºS, whose ecological niche is still unknown. The infection is contracted through the inhalation of airborne propagules from the mycelium phase of the fungus, which soon turn into the pathogenic form of yeast prompted by the human body temperature [5,7,8]. The yeast form is generally between 2 and 10 µm in diameter, sometimes reaching 30 µm or even exceeding this size, which in turn is determined by the developmental phase in which it is found. Therefore, small microorganisms are characteristic of the fast proliferation of the pathogen [6].

Inhalation of *Paracoccidioides brasiliensis* by itself leads to infection, even without any active disease manifestation. Disease development depends on the microorganism's virulence [2,5,7] and on the hormonal, genetic, nutritional and immune factors of the host at the moment of the infection or at the reactivation of latent foci. The latency period is variable [7], with reports of it being as long as 60 years [6].

In endemic areas, a large number of people can be infected, but just a minority of them [9], often composed of adult men with agricultural work activity, develop the disease. It is estimated that 10 million people are infected with the fungus, but only 2% of them develop active disease [5-7]. Interhuman transmission does not occur, since in the human body, the fungus assumes the yeast form, which, although parasitic and pathogenic because of the presence of alpha-1-3-glucan and gp43 protease in its cell wall, is not infectious like the mycelial form found in nature [10,11].

Clinical features

Paracoccidioidomycosis is a pyogenic granulomatous process, usually with chronic evolution, which can manifest as dry cough, with progressive production of secretions and dyspnea during physical activity. Lungs, upper airway tract, lymph nodes, skin, oral

mucosa, adrenal glands and digestive tract are often affected [7,12]. Manifestations can differ according to sex, age, genetic factors and immunity of the host. Clinical classification includes paracoccidioidomycosis-infection, as well as the forms acute/subacute, chronic and residual (sequelae) [5,7,13]. Lung compromise can be observed by means of chest X-ray, which shows bilateral and symmetrical reticulonodular infiltrate in the middle third of the lungs [14].

Paracoccidioidomycosis-infection affects healthy individuals who live in the endemic region without any preference for sex or age. Although the chest X-ray can exhibit lung scars, there is no immune response damage in this form. The acute/subacute form is also called juvenile paracoccidioidomycosis, because it affects young people, both males and females at the same rate. It is the most severe form of the infection, with suppression of cellular immune response and increased specific antibody population. In this form (acute/subacute/juvenile), the development is even faster, and liver, spleen, bone marrow and lymph nodes are compromised. Chronic paracoccidioidomycosis, on the other hand, occurs preferably in males, older than 30 years, with prolonged course and slow and gradual onset. It can be unifocal, affecting just one organ or system, or multifocal when affecting more than one site (skin, mucosae, lungs, adrenal glands). Residual or sequelae forms include signs and symptoms related to scars of old lesions. Chronic pulmonary insufficiency caused by fibrosis is the most severe sequela of the disease [9,12,13,15]. In general, just two classifications are used: acute or juvenile paracoccidioidomycosis and chronic or adult paracoccidioidomycosis, each one with different levels of cellular immunodeficiency [3,16].

Hematogenic dissemination of the fungus from the lungs can originate secondary lesions in oral [6], rectal and intestinal mucosae and skin [11]. Oral lesions are of slow evolution [6,11,17] and multifocal behavior [6], compromising the tongue, floor of the

mouth, alveolar mucosa, gingiva, palate, lips, oropharynx and buccal mucosa [6,11,17]. They manifest as granular ulcers with hemorrhagic dots, where the condition is called mulberry stomatitis [6,11] (Fig.1). Periodontal involvement can also be observed. Gingiva can be erythematous and edematous, and tissue destruction can result in periodontal bone loss, with exposed tooth root, tooth mobility and loss, similar to severe periodontitis [18]. Hard palate perforation, although rare, can occur [6,11]. It is also possible to see macrocheilia [11,17], characterized by the swelling of the lips [17] (Fig.1d). In the acute disseminated form, lymph nodes become enlarged, firm to palpation and coalescent, and they can show fistulas with pus drainage [6,11]. Otherwise, in chronic paracoccidioidomycosis, lymph node swelling in submandibular and cervical chains can be associated with the characteristic oral lesions [6,19].

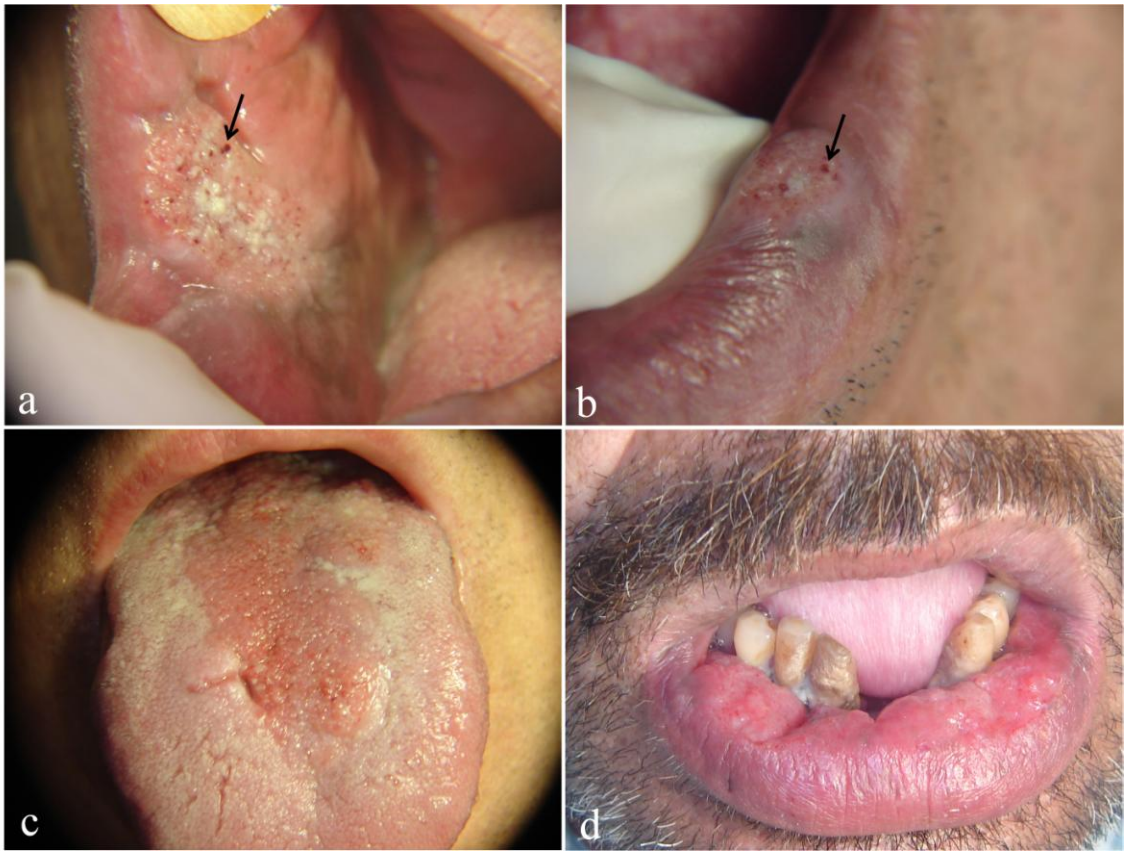

Figure 1 – Oral lesions of paracoccidioidomycosis: mulberry stomatitis showing hemorrhagic dots in retrocomissural mucosa (a), lower lip (b) and dorsum of the tongue (c). Macrocheilia (d): swelling of the lower lip affected by ulcerated lesions.

Histopathology

Inflammatory response to *Paracoccidioides brasiliensis* is represented by epithelioid granuloma, which constitutes a specific immune response against the fungus to prevent its dissemination. This structure is formed of macrophages that surround the pathogen, mature, and differentiate into cells with epithelial appearance. Granulomas can be compact or loose. The compact ones are characterized by densely aggregated epithelioid cells with the fungus inside, seen in the more localized forms of the disease. The loose granulomas show greater amounts of inflammatory exudate, edema, necrosis and fungus, these being characteristic of the more severe cases [5,12,20].

On hematoxylin-eosin examination (H&E, Fig. 2), ulceration of the overlying epithelium, pseudoepitheliomatous hyperplasia and the peculiar granulomatous structure composed of epithelioid macrophages and multinucleated giant cells are observed. The fungus can be found inside the giant cells or free within the tissues showing budding yeast cells with multiple narrow-based buds (adhering to the mother cell), resembling *Mickey Mouse ears* or a *pilot wheel* [5].

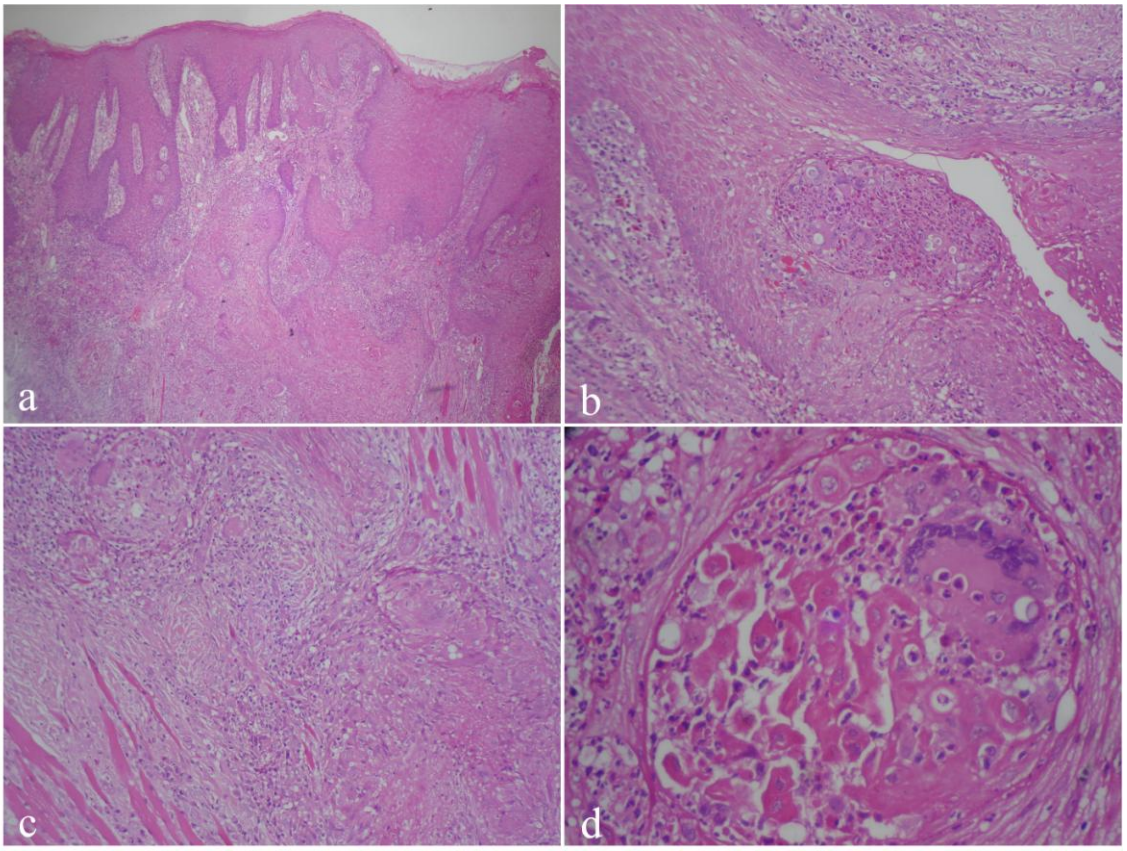

Figure 2 – Histopathological features of oral lesions of paracoccidioidomycosis on hematoxylin-eosin staining: pseudoepitheliomatous hyperplasia (**a** x100, **b** x200), granuloma showing epithelioid macrophages and giant multinucleated cells (**c** x200) with *Paracoccidioides brasiliensis* yeast inside, and microabscesses (**d** x400).

Immunology

The 43-kDa glycoprotein found in the fungus cell wall can induce different responses in the host [21]. A considerable number of healthy people living in the endemic zone show a positive intradermal reaction with paracoccidioidin, without developing the disease, which denotes the preservation of cellular immune response [11]. On the other hand, patients with severe paracoccidioidomycosis can test negative to paracoccidioidin because of immunodeficiency [6].

Severity and clinical presentation of the disease depend on the cellular immune response, where deficiency implies unfavorable prognosis [1,16]. The mechanism by which the host defends itself from *Paracoccidioides brasiliensis* involves Th1

lymphocytes, by means of delayed type hypersensitivity [22], whose efficacy prevents fungal dissemination through the development of granulomas [23]. These are composed of epithelioid cells, macrophages and lymphocytes. A granuloma constitutes a primitive response based on the phagocytosis and removal of the persistent pathogens and irritants [24]. Phagocytes and lymphocytes play a major role in host defense against infection by secreting cytokines, which prevent fungal dissemination to other organs and tissues [16]. Benign or localized paracoccidioidomycosis is the result of cellular immune response Th1 type with IFN-gamma, IL2 and TNF-alpha production and low levels of IL4, IL5, IL10 and antibodies. Disseminated disease shows Th2 type cellular immune response with high levels of IL4, IL5, IL10 and antibodies, B cell polyclonal activation and impairment of IFN-gamma production [2,25].

Therefore, immune system failure in paracoccidioidomycosis seems to result from an imbalance in cytokine production [16,21], which along with lymphopenia is a characteristic feature of the disease [16]. At diagnosis, lymphopenia can be observed because of the reduction in CD4 cells, which are responsible for IL-2 production, which in turn induces IFN-gamma production. The latter mediates macrophage activation and TNF-alpha production, as well as intracellular killing of the fungus [1]. Thus, reduction in IL-2 and IFN-gamma serum levels is related to the severity of the disease [1,5,7], with elevated levels of TNF-alpha being associated with severe symptoms such as anorexia, fever and excessive weight loss. Also, its intense production together with TGF-beta production results in fibrosis of the affected organs through collagen fiber deposition, especially in the lungs [9,12,15].

Although high antibody levels have been detected in the serum of infected individuals, it is known that humoral response plays a coadjuvant role in host defense, favoring the complement system activation and facilitating pathogen phagocytosis

[9,12,15]. In severely compromised patients, strong activation of B cells, hypergammaglobulinemia and increased titers of specific antibodies are associated with severity and dissemination of the disease [1,5,7].

Rats infected with *Paracoccidioides brasiliensis* by the intrathoracic route showed worse immune response with long-lasting infection. Compact granulomas with fungi inside were characteristic of the initial phase of the infection, but in the late phase, this feature was lost with inability of the structure to prevent fungal dissemination. These events were associated with reduction of polymorphonuclear cells, IFN-gamma and nitric oxide (NO), consequent to IL-10 synthesis, which is an imbalance capable of making the animal more susceptible to the disease [15].

Immune response tends to be compromised in malnourished patients infected with *Paracoccidioides brasiliensis*. Studies report that inadequate intake of protein results in higher predisposition to infections because of the impairment of cytokine performance [26,27]. Older rats subjected to high protein diet showed impairment of both proliferation and cytotoxic capacity of leukocytes [28]. Oarada *et al*. [27] evaluated diet protein concentration that induced the best immune response to *Paracoccidioides brasilienses* in rats. The authors observed that rats fed high-protein levels in a short period of time had impaired immune response when compared to rats that were fed normal levels of protein. The spleen and liver of animals treated with high-protein diet showed increased levels of IFN-gamma and retarded antifungal activity when compared to animals treated with low protein amounts. As the increase in production of IFN-gamma and proinflammatory cytokines contributes to pathogen elimination and is related to the time the agressor agent stays in the host, the increase in IFN-gamma production in rats under high-protein diet could have happened because of the longer time required for pathogen elimination.

The immune system in paracoccidioidomycosis patients is also impaired by alcoholism. Studies report that alcohol abuse is a predisposing factor to deep mycoses because of the malnutrition and immunosuppression it causes. Alcoholism influences chronic paracoccidioidomycosis pathogenesis, where most of these patients drink alcohol in great amounts for long periods [29]. Often, patients combine smoking with drinking, which potentiates susceptibility to the disease [30].

Nicotine depresses immunity by stimulating Th2 cells to synthesize high levels of IL4, which acts in Th1 cells preventing proinflammatory cytokine production [31]. The inhibitory effect of nicotine involves the activation of alpha-7-nicotinic acetylcholine receptor present in macrophages, T and B cells, which lowers the synthesis of TNF-alpha, IL-1 beta and IL-6 proinflammatory proteins. In this case, suppression of Th1 response occurs without impairment of Th2 [32]. Therefore, an imbalance in the Th1/Th2 ratio favors infection by pathogens [31] such as *Paracoccidioides brasiliensis*.

In AIDS patients, paracoccidioidomycosis can result from the reactivation of quiescent foci, resembling the acute form of the disease [11,33], with phagocytic mononuclear involvement [11]. Most common clinicial manifestations are generalized lymphadenopathy, splenomegaly, fever, weight loss, skin lesions, and pulmonary and neurologic injury [34]. Associated with this, mucosal lesions, which are characteristic of chronic paracoccidioidomycosis, can also be found in coinfection cases, giving them the name *mixed form* [35]. Patients coinfected with *Paracoccidioides brasiliensis* and HIV show lower titers of specific antibodies when compared to patients with only paracoccidioidomycosis. This finding points out the lower specific humoral response intensity, which can be explained by the B cell dysfunction associated with HIV and possibly by the rapid progression of paracoccidioidomycosis in these cases [36]. Severe immunosuppression with low levels of CD4 favors the establishment of

paracoccidioidomycosis [11,36], which can be the first sign of HIV immunosuppression [11]. Therefore, simultaneous manifestation of these two diseases is associated with a high mortality rate [37]. Nevertheless, coinfection rates are low, probably because AIDS patients are often under prophylaxis with drugs routinely used in the treatment of paracoccidioidomycosis such as sulfonamides and azole derivatives [35]. Besides, epidemiological differences between these diseases, where AIDS is not as frequent in rural zones as is paracoccidioidomycosis, also account for these low rates of coinfection [11,19,33,36].

Women have a significantly lower prevalence of paracoccidioidomycosis, where they are protected against *Paracoccidioides brasiliensis* by the female hormone 17-beta-estradiol. The interaction of this hormone with the receptor in the cytosol of the fungus prevents its transition from mycelium to yeast, which blocks the disease onset [11,38,39]. In these cases, the estradiol mechanism of action can be related to the modulation of the expression of genes that regulate fungal dimorphism, determining features such as cell wall maintenance and remodeling, energy metabolism and fungal response to temperature changes, among others [38].

Diagnostic methods

Direct microscopic examination or direct mycological examination is used to identify *Paracoccidioides brasiliensis* in purulent discharge of lymph nodes, sputum or material collected from the lesions [11,40]. Culture of the affected tissues can also be used in the diagnosis [6,11,40,41], but it is hampered by the very slow fungal growth [40,41]. It is still possible to visualize fungal particles in the cytopathological and histopathological examinations through Gomori-Grocott (Fig. 3 a, b) and PAS (periodic acid-Schiff) (Fig. 3 c, d) staining [6,14,40]. Exfoliative cytology is a non-invasive and low-cost method, which can help the diagnosis, especially if associated with silver staining in the Gomori-Grocott

technique. This provides fast and easy visualization of *Paracoccidioides brasiliensis* and can be used for monitoring the infection during and after treatment [42].

Serological methods can also be used to confirm diagnosis and monitor therapy [11,17,40]. The principal component recognized in serological examinations is a 43-kDa glycoprotein, the major antigen of *Paracoccidioides brasiliensis*, which is secreted during fungal infection and identified in serum of all patients with the disease. Antibodies are produced against this antigen and can also be used in the diagnosis and monitoring of the response to treatment [11]. The serological techniques most used in paracoccidioidomycosis are ELISA, counterimmunoelectrophoresis and double immunodiffusion, whose use has been based on their high sensitivity and specificity, as well as simple methodology and reasonable cost [11,40].

The search for a faster and precise diagnostic method has prompted the use of highly sensitive and specific techniques. *Polymerase chain reaction* (PCR) and immunohistochemistry are applied when serology and histopathology are inconclusive [6]. The use of monoclonal antibodies such as MAbs PS14 and MAbs PS15 directed at a glycoprotein with molecular mass between 22 and 25 kDa found in *Paracoccidioides brasiliensis* has been studied as a potential alternative for the confirmation of diagnosis through immunohistochemistry [41].

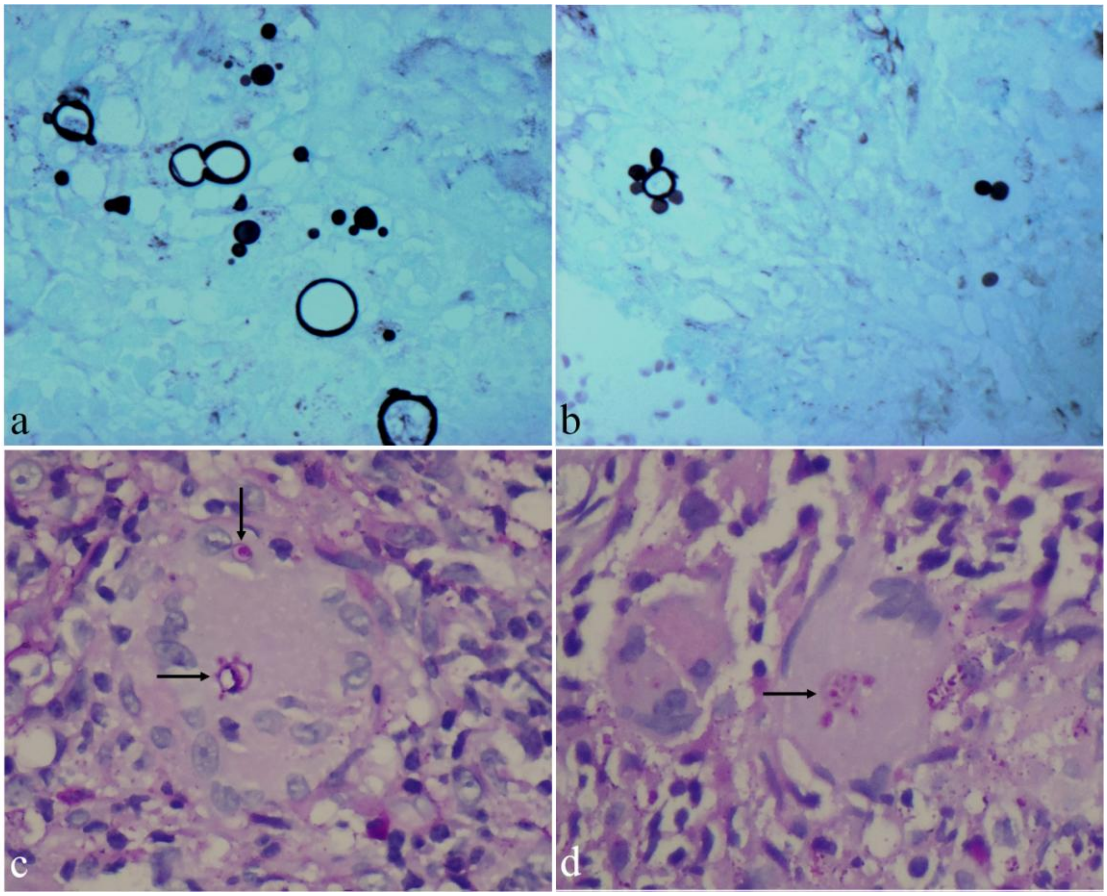

Figure 3- *Paracoccidioides brasiliensis* detected by histopathological examination of oral lesions using Gomori-Grocott (x400; a, b) and PAS (x400; c, d) staining. It is possible to see the characteristic multiple buds resembling a *pilot wheel.*

Differential diagnosis

Oral squamous cell carcinoma is the main differential diagnosis in oral paracoccidioidomycosis [6,11,40], because of the similar clinical aspects and the association of both diseases with alcohol and tobacco. Discerning features such as pain and multifocal lesions in paracoccidioidomycosis, which are not common in squamous cell carcinoma can help clinical diagnosis [14]. Moreover, oral lesions of hystoplasmosis, syphilis, tuberculosis [6,11,40], Wegener's granulomatosis [6], leishmaniasis [6,11,40], sarcoidosis [11], lymphoma [11,40] and actinomycosis [40] also mimic paracoccidioidomycosis and should be considered.

Treatment

If not adequately treated, paracoccidioidomycosis can be fatal. Treatment requires drug therapy with loading doses, nutritional support, management of sequelae, and maintainance of the patient in good health with rigorous follow-up [11]. The choice of drug and treatment duration will depend on the severity of the disease [11,17,40]. Itraconazole, a member of the azole drug group, is indicated in mild and moderate cases of either acute or chronic paracoccidioidomycosis. Ketoconazole is an alternative treatment, but it is not the first-choice drug, because of its important side effects when used for extended periods. Sulfonamides were the first drugs used for paracoccidioidomycosis treatment. They show good results and are inexpensive when compared to other drugs; however, extended treatment time is required with many daily administrations, and they are contraindicated in cases of sulfonamide hypersensitivity [40]. Voriconazole is a broad-spectrum triazole antifungal drug, whose efficacy is similar to that of itraconazole [17,40], with a stronger effect on the central nervous system. Therefore, it is indicated especially in neuroparacoccidioidomycosis, even though its elevated cost is a disadvantage compared to the other drugs [11]. Amphotericin B belongs to polyenic antibiotics and is indicated in severe cases. Toxicity and side effects are inherent to its intravenous administration, requiring patient hospitalization [11,40]. Inappetence, fever, nausea, chills, phlebitis of the vein used for drug administration, tachycardia and hypertension are mild side effects, which can be reversed with corticosteroids [11,17]. As nephrotoxicity is an important side effect, monitoring of renal function is crucial in these patients [17].

Considering the long-term treatment, which can last from 6 to 24 months [17], and the possibility of recurrence, new therapeutic options are needed for paracoccidioidomycosis. A vaccine development from gp43 may be an alternative to be used in combination with regular drug therapy [40]. Cure should consider clinical,

radiographic and immune criteria. Regression of the lesions and elimination of the characteristic signs and symptoms, chest imaging showing stabilization of the lesions during follow-up, and serological tests with low antibody titers are criteria to be analyzed before determining the cure of the patient [11,14].

Final considerations

Paracoccidioidomycosis is a systemic disease with an endemic profile and considerable morbidity and mortality. Clinical manifestations include oral lesions, which are often the major sign and site of confirmation of diagnosis. The diagnosis, in turn, is delayed in most cases. Success of the treatment depends on early and correct diagnosis, as well as on the patient's adherence to the drug therapy, which lasts for extended periods of time. The possibility of death or severe sequelae such as pulmonary fibrosis does exist. It is also important to pay attention to population aging in endemic zones and increasing migration rates from rural areas to large urban centers, as immune system diseases can favor reactivation of infectious foci after many years of latency.

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3 ARTIGO 2

O artigo a seguir intitula-se **Interrelationship of clinical, histomorphometric and immunohistochemical features of oral lesions in chronic paracoccidioidomycosis** e foi formatado de acordo com as normas do periódico *Journal of Oral Pathology and Medicine* (Anexos C e D).

Interrelationship of clinical, histomorphometric and immunohistochemical features of oral lesions in chronic paracoccidioidomycosis

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Running title: *Oral paracoccidioidomycosis*

Keywords: paracoccidioidomycosis; interleukin-2; tumor necrosis factor-alpha; interferon-gamma; *Paracoccidioides brasiliensis*

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ABSTRACT

BACKGROUND: This study aimed to analyze the oral lesions of chronic paracoccidioidomycosis concerning their histomorphometric, immunohistochemical and clinical features in a standardized sample.

METHODS: Fifty biopsy specimens of oral lesions of chronic paracoccidioidomycosis were submitted to hematoxylin and eosin (H&E), Gomori-Grocott and immunohistochemical staining. Data regarding disease duration and size and number of oral lesions, as well as erythrocytes, leukocytes, lymphocytes, hematocrit, hemoglobin and erythrocyte sedimentation rate, were collected from medical charts. Granuloma density and number and diameter of buds and fungal cells, and IL-2, TNF-alpha and IFN-gamma expression, as well as clinical and hematological features, were quantified and correlated.

RESULTS: Bud diameter was significantly greater in intermediate density granulomas compared to higher density granulomas. The other variables (number of buds, number and diameter of fungi, expression of IL-2, TNF-alpha and IFN-gamma, and clinical and hematological features) did not significantly change with the density of granulomas. There was a positive correlation between bud number and fungal cell number (r=0.834), bud diameter and fungal cell diameter (r=0.496), erythrocytes and number of fungi (r=0.420), erythrocytes and bud number (r=0.408), and leukocytes and bud number (r=0.396). Negative correlation occurred between number and diameter of fungi (r=-0.419), bud diameter and granuloma density (r=-0.367), TNF-alpha expression and number of fungi (r=-0.372), and TNF-alpha expression and bud number (r=-0.300).

CONCLUSIONS: The histological, immunological and clinical features of oral lesions evaluated did not differ significantly between patients in our sample of chronic paracoccidioidomycosis. TNF-alpha levels were inversely correlated with intensity of infection.

Introduction

Paracoccidioidomycosis is a systemic mycosis endemic to some countries of Latin America (1,2). The contagion occurs by inhalation, where the lungs are the first site affected, and, by hematogenous and lymphatic dissemination, other sites can be compromised, including the oral cavity (1-3). The clinical spectrum of the disease can vary from subclinical infection to moderate or severe cases, depending on the immune factors of the host and fungal virulence. The major clinical forms of the disease are acute/subacute and chronic. The acute/subacute form affects children and adolescents, both male and female, and is aggressive and disseminated, whereas the chronic form is more localized, commonly with pulmonary and mucocutaneous involvement, and affects mainly men (90% of cases) aged between 30 and 60 years old (4,5). Most patients with chronic paracoccidioidomycosis are tobacco and alcohol users, often showing chronic obstructive pulmonary disease, which can delay the diagnosis (4). Therefore, oral lesions are many times the target of the diagnostic investigation.

On clinical examination, oral lesions are yellow ulcers or erosions with a granulated surface and pinpoint red dots showing tiny hemorrhagic areas. On histological examination, it is possible to see the typical granulomatous structure with epithelioid macrophages and multinucleated giant cells, and the overlying epithelium showing an ulcerated surface as well as pseudoepitheliomatous hyperplasia. Fungi are observed inside the multinucleated giant cells or free within the tissues with multiple buds adhered to the mother cell (4,6). Some studies have demonstrated a relationship between the histological architecture of the lesion and the host immune response to *Paracoccidioides brasiliensis (P. brasiliensis).* This response is represented by the epithelioid granuloma, which is a specific reaction against the fungus that prevents its dissemination. Granulomas can be dense or loose. Dense granulomas are characterized by densely aggregated epithelioid cells

with the fungus inside and represent more localized forms of the disease. Loose granulomas have greater amounts of inflammatory exudate, edema and necrosis and greater number of fungi, and are characteristic of more severe cases (7,8).

The diagnosis of paracoccidioidomycosis by means of hematoxylin and eosin (H&E) staining can be impaired by the difficulty in visualizing the fungus with this technique, and thus, Gomori-Grocott staining is used to better identify the pathogen in the tissues. Moreover, there are few reports in the literature about the use of immunohistochemistry in paracoccidioidomycosis diagnosis (4,9). Figueroa *et al*. (9) produced specific antibodies, MAbs PS14 and PS15, against *P. brasiliensis*, which can be applied in immunohistochemistry as a diagnosis tool. Some studies have also investigated the role of interleukin-2 (IL-2), tumor necrosis factor-alpha (TNF-alpha) and interferon-gamma (IFN-gamma) in paracoccidioidomycosis. The function of these cytokines is related to the efficacious immune response of the host. IL-2, produced by T CD4 lymphocyte, induces IFN-gamma, which is responsible for TNF-alpha production. IFN-gamma and TNF-alpha have important roles in host resistance against the fungal infection, granuloma formation and control of pathogen dissemination. Therefore, the expression of these cytokines in oral lesions could be used as a prognostic factor of the disease (2,10).

The literature lacks studies focusing on histological features of oral lesions of paracoccidioidomycosis and their relationship with immune aspects and the clinical picture of the patients. This work aimed to analyze oral lesions of chronic paracoccidioidomycosis concerning their histomorphometric, immunohistochemical and clinical features in a standardized sample. The density of granulomas, fungal morphometry, and IL-2, TNF-alpha and IFN-gamma immunohistochemical expression, as well as clinical and hematological features, were analyzed and correlated.

Material and methods

This study was approved by the Ethics Research Committee of Pontifical Catholic University of Rio Grande do Sul. The sample comprised medical charts and paraffin blocks of biopsied specimens both from 50 adult patients presenting with oral lesions of chronic paracoccidioidomycosis. Forty-six patients were males, 4 patients were females, and no immunocompromising diseases were recorded among them. The age ranged between 29 and 75 years old, and all were smokers. Inclusion criteria were medical chart adequately filled and paraffin block in good conditions for histological analysis. Data concerning (a) duration of the disease, (b) number and size of oral lesions, and (c) hematological parameters before treatment (erythrocytes, hematocrit, hemoglobin, leukocytes, lymphocytes and erythrocyte sedimentation rate) were collected. The paraffin blocks were submitted to histological processing.

Histological processing

Four micrometer-thick histological cuts were obtained from the specimens and submitted to H&E and Gomori-Grocott staining. These slides were reviewed to confirm the diagnosis according to previously reported histopathological criteria (7,8). On H&E examination, the criteria included: granulomatous formation composed of epithelioid macrophages and multinucleated giant cells with fungi inside the giant cells or dispersed within the tissues; edema; and necrosis and microabscesses. On Gomori-Grocott examination, diagnosis confirmation was established based on the presence of fungi with multiple buds adhered to the mother cell. As the diagnosis was confirmed, the sample was submitted to immunohistochemistry.

Immunohistochemical processing

Immunohistochemistry was based on the streptavidin-biotin-peroxidase technique. Three micrometer-thick sections were obtained, placed on slides pretreated with Histogrip (Zymed, Carlsbad, CA, USA) and allowed to stand for 24 h in an electric oven at 60ºC. The sections were deparaffinized, and antigen retrieval was by heat at high temperature under pressure with slides incubated in Coplin jars (Laborglas, Mainz, Rheinland-pfalz, Germany). IL-2 antibody was used with the Dako target retrieval solution, pH 9 (Dako, Carpinteria, CA, USA), where the sections were placed in a water bath at 100 °C for 40 min and cooled for 20 min at room temperature. Anti-IFN-gamma and anti-TNF-alpha were incubated at 37°C with 0.01% trypsin solution for 1 h. Endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol. Nonspecific antibody binding was blocked with Protein Block Serum-Free (Dako). The antibodies used were anti-IL-2 (Novocastra, Newcastle Upon Tyne, NE, UK), anti-IFN-gamma (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-TNF-alpha (Santa Cruz Biotechnology) respectively at dilutions of 1:200; 1:80 and 1:100. Sections were incubated with antibodies diluted in antibody diluent with background reducing components (Dako), using the capillarity method in a Sequenza Immunostaining Center (Thermo Shandon, Pittsburgh, PA, USA) overnight at 2ºC to 6ºC. The antigen-antibody reaction was amplified with the Picture Max system, HRP Polymer Conjugate Broad Spectrum (Invitrogen, Carlsbad, CA, USA). Slides were incubated in the diaminobenzidine Dako Liquid DAB Substrate Chromogen System (Dako), counterstained with Harris hematoxylin and incubated with 37 mM ammonia. Next, they were dehydrated in ethanol, treated in xylene and mounted in Entellan (Merck, Darmstadt, Hessen, Germany). The positive controls were provided by tonsil sections, whereas the omission of the primary antibodies served as the negative controls.

Histological analysis

Histological images were digitized using a light microscope Zeiss Axioskop 40 (Zeiss, Goettingen, Germany), connected by a videocamera CoolSnap Pro (Media Cybernetics, Bethesda, MD, USA) to a microcomputer. Images were stored in Joint Photographic Experts Group (JPEG, Pegasus Imaging Co., Arlington, WA, USA) format, and analyzed with Image Pro Plus 4.5.1 software (Media Cybernetics). On H&E, 6 fields were captured using x10 (3 fields) and x20 (3 fields) objectives; on Gomori-Grocott, 10 fields were captured using a x40 objective; and for immunohistochemistry analysis, 10 fields were captured for each marker using a x40 objective. All captures were made in a standardized manner. Histological analysis was performed by one blinded and calibrated observer. Calibration consisted in analyzing a series of 10 images of each histological technique, twice at different moments. The results of these evaluations were submitted to the Wilcoxon test and Spearman correlation coefficient for H&E and to intraclass correlation for Gomori-Grocott and immunohistochemistry. The results of the tests showed strong correlation and no significant difference between the evaluations.

H&E images were classified according to granuloma density by means of a quantitative analysis. Microabscesses, edema, necrosis, dispersed fungi in the tissues and multinucleated/epithelioid giant cells were analyzed and quantified in each field. Giant cell analysis was based on the scores 0 (absent), 1 (mild), 2 (moderate) and 3 (intense). The analysis of microabscesses, necrosis, edema and dispersed fungi in the tissues was based on the scores 0 (intense), 1 (moderate), 2 (mild) and 3 (absent). The use of the two classifications was based on the inverse relation between giant cells and the other features concerning dense and loose granulomas. That is, loose granulomas are formed mainly by microabscesses, necrosis, edema, and dispersed fungi in the tissues, whereas dense granulomas are composed mainly of giant cells to the detriment of those features. Scores of each field were summed up, resulting in one score for each slide, and the sample was

divided in tertiles, which resulted in 3 groups: lower density granuloma, intermediate density granuloma and higher density granuloma.

Gomori-Grocott images were analyzed by using a specific tool for linear measurements in Image Proplus 4.5.1 (Media Cybernetics, Fig. 1), and the number of fungal cells and their buds, as well as their respective diameters were obtained. In the immunohistochemical images, the immunostained areas were quantified (proportion of area stained) by means of a semiautomated segmentation technique, also in Image Proplus 4.5.1 (Media Cybernetics, Fig.2). In both Gomori-Grocott and immunohistochemistry samples, measurements were obtained from the 10 fields previously selected, and the mean for each slide was calculated (Fig.3).

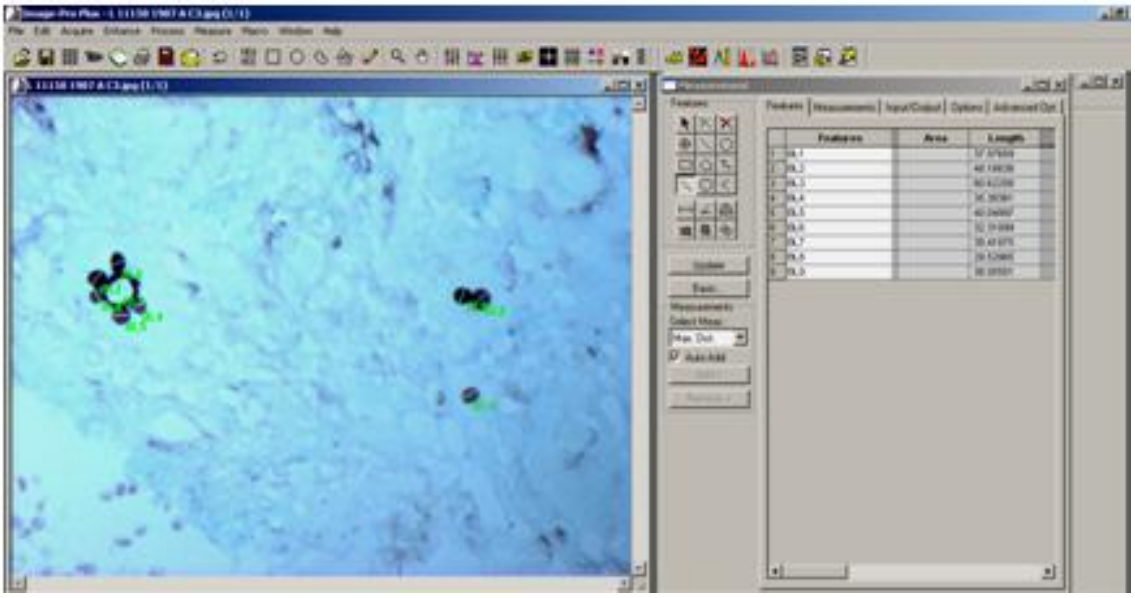

Figure 1- Quantitative analysis in Gomori-Grocott (x400) by using Image Proplus 4.5.1 (Media Cybernetics, Silver Spring, MD, USA)

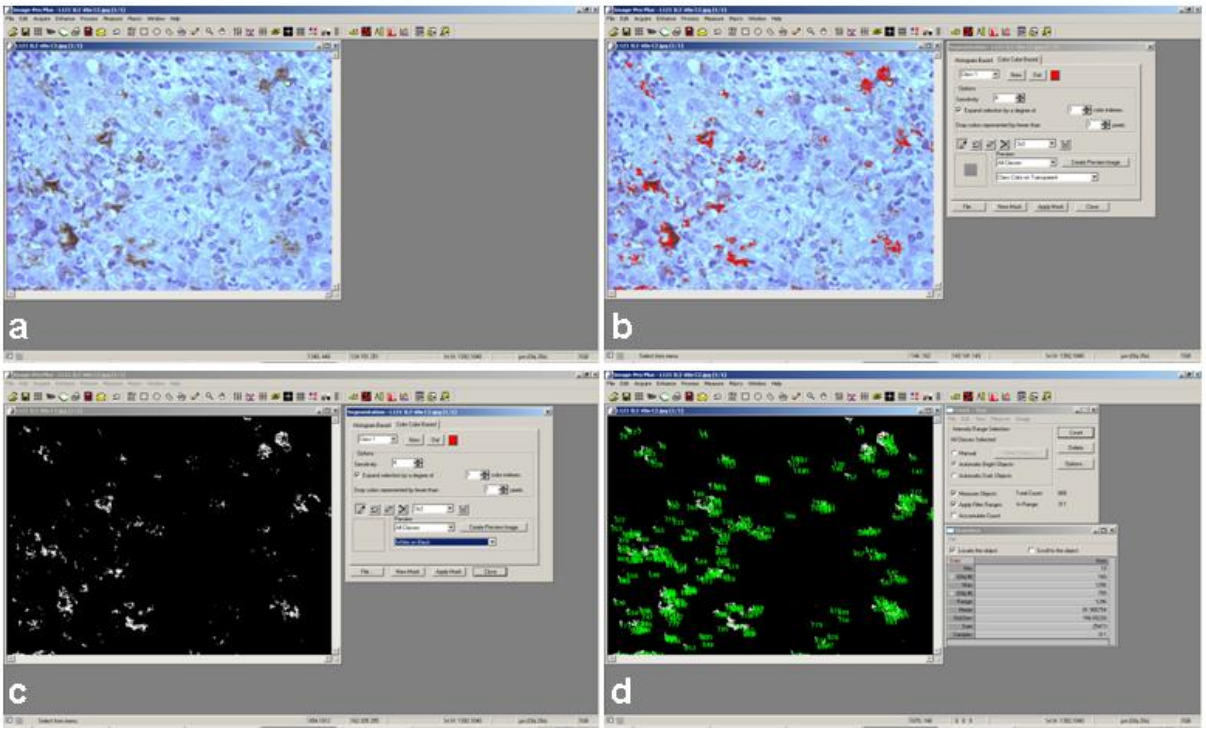

Figure 2 - Analysis of immunostaining for IL-2 by means of semiautomated technique in Image Pro-plus 4.5.1 (Media Cybernetics, Silver Spring, MD, USA)

Statistical analysis

Data were analyzed by means of descriptive statistics, and the variables were compared between the groups of granulomas and also correlated. The comparison of variables with normal distribution was made with ANOVA, which when significant was complemented by the Tukey multiple comparisons test. The variables that had no normal distribution were compared by means of the Kruskal-Wallis test, and the correlation between all the variables was tested using Pearson or Spearman coefficients. Data were processed in SPSS 17.0 (Statistical Package for the Social Sciences, Chicago, IL, USA), considering a significance level of 5%.

Results

Number and diameter of fungal cells and number and diameter of buds

There was no significant difference between the groups for number and diameter of fungal cells and number of buds (Table 1, ANOVA, Kruskal-Wallis, α=0.05). Bud diameter was significantly greater in group 2 when compared to group 3 (*P*=0.011), but this variable did not significantly differ between the other groups (ANOVA, Tukey, α=0.05).

Table 1 – Analysis of number and diameter of fungal cells and number and diameter of buds, according to density of granulomas

	Group 1 (LDG)			Group 2 (IDG)			Group 3 (HDG)			
Variable	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median	*P*
No. of fungi	64.53	63.98	34.0	34.19	17.71	26.5	65.06	51.54	61.0	0.343*
Fungal cell diameter (µm)	26.15	5.48	25.75	26.30	6.66	25.67	22.65	4.42	22.33	0.111**
No. of buds	32.5	36.34	19.0	19.88	15.54	12.5	32.53	29.00	18.0	0.435*
Bud diameter (µm)	15.70^{AB}	3.47	15.57	17.03^{A}	5.58	14.94	12.27^{B}	4.19	12.32	0.011**

LDG= Lower density granuloma; IDG=intermediate density granuloma; HDG=higher density granuloma; *Kruskal-Wallis, α=0.05; **ANOVA, Tukey, α=0.05; for bud diameter, means followed by different letters showed a significant difference

IL-2, TNF-alpha and IFN-gamma

There was no significant difference in immunostaining quantification for IL-2, TNF-alpha and IFN-gamma between the groups analyzed (Table 2, Kruskal-Wallis, α=0.05).

Table 2 – Quantification (%) of immunostaining for IL-2, TNF-alpha and IFN-gamma according to density of granulomas

	Group 1 (LDG)			Group 2 (IDG)			Group 3 (HDG)			
Variable	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median	*P*
IL-2	2.97	3.77	1.7	5.79	8.53	3.0	6.26	9.47	2.3	0.316*
TNF-alpha	12.19	6.31	11.4	14.87	8.93	13.1	12.61	7.03	12.5	0.746*
IFN-gamma	4.42	3.00	4.2	11.25	14.75	5.3	6.81	5.30	6.8	0.426*

LDG= Lower density granuloma; IDG=intermediate density granuloma; HDG=higher density granuloma; *Kruskal-Wallis, α=0.05

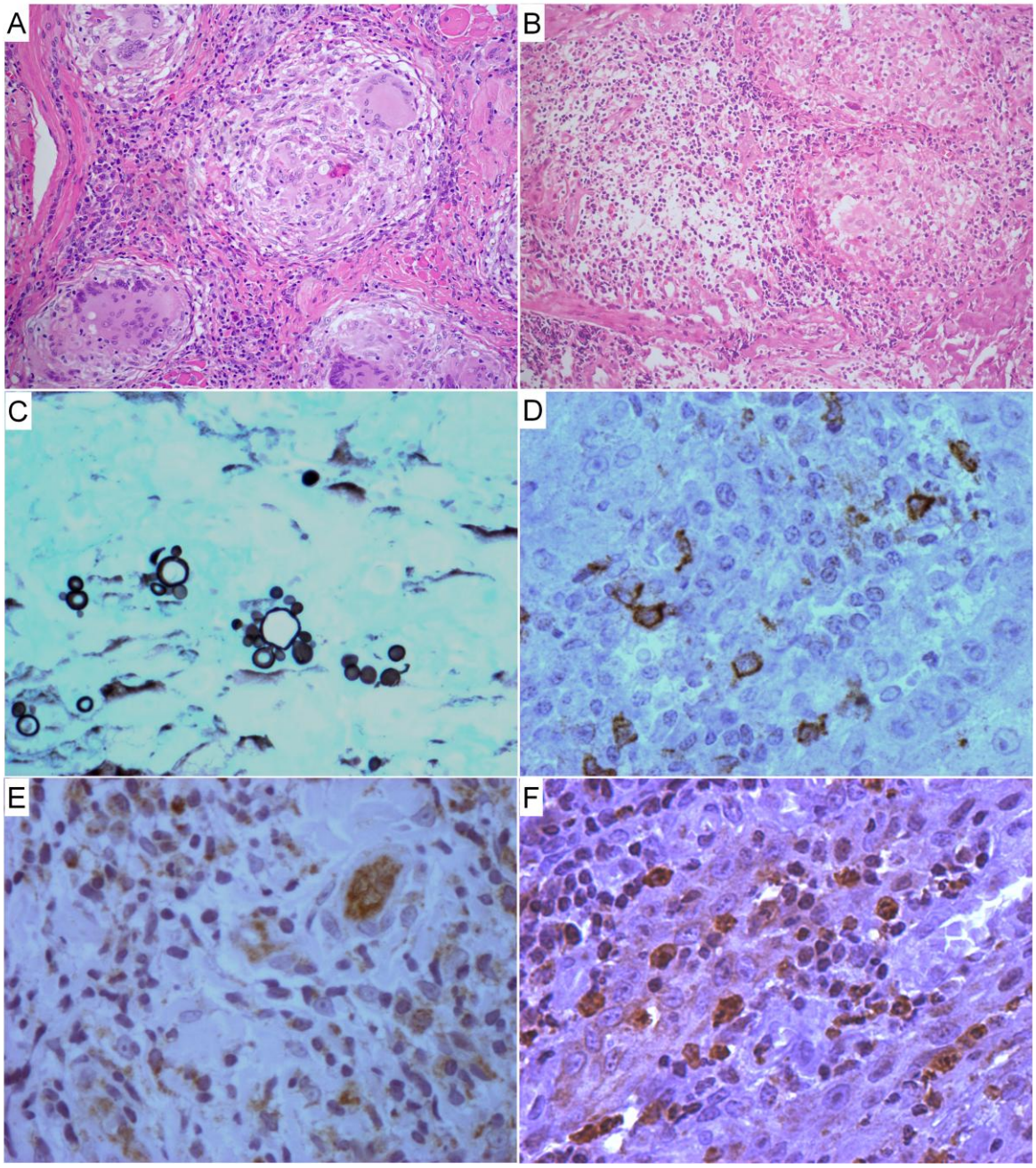

Figure 3 – Microscopic features of oral lesions in chronic paracoccidioidomycosis. High (A) and low (B) density granuloma in hematoxylin and eosin, x200; Gomori-Grocott staining, x400, evidencing yeast cells with multiple buds (C); Positive immunostaining, x400 for IL-2 (D), TNF-alpha (E) and IFN-gamma (F).

Hematological parameters

The hematological parameters (erythrocytes, hematocrit, hemoglobin, leukocytes, lymphocytes and erythrocyte sedimentation rate) did not significantly differ between the groups (Table 3, ANOVA, Kruskal-Wallis, $p>0.05$).

Table 3 – Analysis of hematological parameters according to density of granulomas

Variable	Group 1 (LDG)			Group 2 (IDG)			Group 3 (HDG)			*P*
	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median	
Erythrocytes ($x10^6$/µL)	4.92	0.31	4.91	4.76	0.51	4.79	4.7	0.49	4.91	0.501*
Hematocrit (%)	42.58	4.69	44	44.12	3.46	45.25	43.4	3.85	43.70	0.672*
Hemoglobin (g/dL)	14.35	1.01	14.30	14.62	1.29	14.74	14.4	1.32	14.10	0.875*
Leukocytes ($x10^3$/ µL)	8.97	2.45	8.78	7.08	1.75	7.3	8.12	2.79	7.8	0.155*
Lymphocytes ($x10^3$/µL)	1.79	0.518	1.79	1.82	0.493	1.70	2.07	0.815	2.14	0.614*
ESR (mm/1st h)	22.25	13.52	19.0	29.57	22.55	21.5	38.75	30.49	29.0	0.503**

LDG= Lower density granuloma; IDG=intermediate density granuloma; HDG=higher density granuloma; ESR= erythrocyte sedimentation rate; *ANOVA, α=0.05; **Kruskal-Wallis, α=0.05

Clinical features

Duration, number and mean size of the lesions did not show any significant difference between the groups analyzed (Table 4, Kruskal-Wallis, *P*>0.05).

Table 4 – Analysis of clinical features according to density of granulomas

Variable	Group 1 (LDG)			Group 2 (IDG)			Group 3 (HDG)			*P**
	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median	
Duration (months)	8.41	10.83	5.0	6.21	4.82	5.0	5.94	7.10	3.0	0.621
No. of lesions	3.0	2.34	2.0	3.88	2.02	4.0	2.53	1.28	2.0	0.126
Size of lesions (cm)	2.47	1.12	2.4	2.54	1.70	2.3	2.77	1.39	3.0	0.469

LDG= Lower density granuloma; IDG=intermediate density granuloma; HDG=higher density granuloma; *Kruskal-Wallis, α=0.05

Correlation analysis (Table 5)

Considering a significance level of 5%, there was a positive correlation between bud number and fungal cell number (r=0.834), bud diameter and fungal cell diameter

(r=0.496), erythrocytes and number of fungal cells (r=0.420), erythrocytes and bud number (r=0.408), and leukocytes and bud number (r=0.396). There was a negative correlation between number and diameter of fungal cells (r=-0.419), bud diameter and granuloma density (r=-0.367), TNF-alpha expression and number of fungal cells (r=-0.372), and TNF-alpha expression and bud number (r=-0.300).

Table 5 –"r" values in correlation analysis between the variables using Spearman and Pearson coefficients

	ESR	GD	No. of fungi	Fungal cell diameter	No. of buds	Bud diameter	IL-2	TNF-alpha	IFN-gamma	Duration	No. of lesions	Size of lesions	ERYT	HT	HB	LEUK	LYMPH
ESR	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GD	0.190	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
No. of fungi	0.045	0.001	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fungal cell diameter	0.101	-0.270	**-0.419***	1	-	-	-	-	-	-	-	-	-	-	-	-	-
No. of buds	-0.146	-0.040	**0.834***	-0.254	1	-	-	-	-	-	-	-	-	-	-	-	-
Bud diameter	-0.128	**-0.367***	0.047	**0.496***	0.144	1	-	-	-	-	-	-	-	-	-	-	-
IL-2	0.305	0.084	0.101	-0.035	-0.153	-0.142	1	-	-	-	-	-	-	-	-	-	-
TNF-alpha	-0.290	0.045	**-0.372***	-0.037	**-0.300***	-0.047	-0.050	1	-	-	-	-	-	-	-	-	-
IFN-gamma	-0.100	0.084	-0.012	-0.203	-0.195	-0.064	0.275	0.065	1	-	-	-	-	-	-	-	-
Duration	0.030	-0.201	-0.168	0.071	-0.083	0.122	-0.010	0.052	-0.178	1	-	-	-	-	-	-	-
No. of lesions	-0.101	-0.052	0.074	0.074	-0.007	0.035	0.147	0.025	-0.034	0.274	1	-	-	-	-	-	-
Size of lesions	0.272	0.154	-0.193	0.195	-0.109	0.093	-0.164	-0.011	0.104	0.113	-0.153	1	-	-	-	-	-
ERYT	**-0.393***	-0.092	**0.420***	-0.162	**0.408***	0.085	-0.059	0.057	-0.072	-0.064	-0.069	-0.146	1	-	-	-	-
HT	**-0.469****	0.082	0.308	-0.212	0.313	0.109	-0.153	0.322	-0.052	-0.355*	-0.163	-0.161	**0.712***	1	-	-	-
HB	**-0.540****	0.084	0.291	-0.194	0.321	0.089	-0.240	0.382*	-0.006	-0.283	-0.198	-0.028	**0.648***	**0.951***	1	-	-
LEUK	-0.136	-0.167	0.279	-0.009	**0.396***	-0.017	-0.146	-0.049	-0.154	-0.003	0.091	-0.172	**0.468***	0.277	0.192	1	-
LYMPH	-0.134	0.211	0.284	-0.202	0.347	-0.280	0.010	0.072	0.286	-0.098	0.189	-0.010	**0.552***	0.329	0.265	**0.572***	1

* Bold printed values show significant correlation, considering $P < 0.05$

ESR= erythrocyte sedimentation rate; GD=granuloma density; ERYT=erythrocytes; HT=hematocrit; HB=hemoglobin; LEUK=leukocytes; LYMPH=lymphocytes

Discussion

Bud diameter was the only variable that showed a significant difference between the groups according to the density of the granulomas, where it was significantly greater in the intermediate density group (group 2) when compared to the higher density group (group 3). According to the literature, the smaller-sized yeast cells are associated with a more intense proliferation of the *P. brasiliensis* (11) and, therefore, with less dense granulomas (7,8). Such observation agrees with our finding (although not statistically significant) of greater bud diameter in group 2 (intermediate density) when compared to group 1 (lower density), but it disagrees with the significant difference observed between the groups 2 and 3. The other variables analyzed did not significantly differ between the density levels of granulomas. Regarding these findings, two points should be considered: (a) the relatively small size of the sample might have contributed to the lack of statistically significant results; or (b) actually the patients with chronic paracoccidioidomycosis in our sample did not significantly differ from each other in clinical, immunological and histomorphometric features of the disease. Anyway, despite a lack of statistical significance, IL-2 levels were higher in group 3 (higher density granulomas). This agrees with the literature, which reports that the increase in levels of this interleukin, as well as IFN-gamma and TNF-alpha, is associated with the resistance of the host against infection with *P. brasiliensis* (12-14). Nevertheless, IFN-gamma and TNF-alpha levels were higher in group 2 (intermediate density), and we do not have a clear justification for this finding. Fornari *et al*. (15) found higher levels of IL-2 and TNF-alpha in serum of chronic paracoccidioidomycosis patients compared to controls, but these authors did not find a significant difference for IFN-gamma. Marques Mello *et al*. (16), in turn, did not find a significant difference for TNF-alpha produced by peripheral blood mononuclear cells according to the clinical severity of the disease.

Maybe an explanation for these disagreements could be the different methods applied, especially considering that most reports about the behavior of these cytokines in paracoccidioidomycosis refer to their serum levels (15-18), whereas we evaluated them in biopsied specimens from oral lesions of patients. Therefore, our results lacking significant changes in the evaluated cytokines could suggest that the serum changes reported in the literature result from the mobilization of the immune cells from the bone marrow and blood into the lesion sites (redistribution) (10) but that it does not represent the level of the cytokines produced at these sites. On the other hand, although murine and clinical models of paracoccidioidomycosis differ in some ways (15), there are reports of an IFN-gamma role in host resistance to *P. brasiliensis* based on histopathological analysis of lung lesions (18,19) and lymph nodes (13) in animal models. These studies showed that IFN-gamma plays a protective role and that this cytokine is a major mediator of resistance against *P. brasiliensis* infection in mice. Moreover, it has been shown that the fungus stimulates the secretion of TNF-alpha by inflammatory cells of chronic paracoccidioidomycosis patients either in peripheral blood or in oral lesions (20), and both acute and chronic paracoccidioidomycosis patients have low levels of IL-2 and IFN-gamma compared to healthy individuals (17).

There seems to be a consensus in the literature about the importance of Th1 immune response type in resistance to *P. brasiliensis*, while Th2 response is associated with the development of the disease. The higher the levels of IL-2, TNF-alpha and IFN-gamma produced in the cellular immune response (Th1 type), the better the response is of the host. On the other hand, the prevalence of the humoral immune response with high levels of IL-4, IL-5, IL-10 and specific antibodies to *P. brasiliensis*, is associated with a poor response to the fungus and more severe cases of the disease (13,16,19). Still, according to Marques Mello *et al*. (16), it is not the significantly low levels of IL-2

and IFN-gamma that are actually the real problem, but the overproduction of IL-4 and IL-5, which are related to the Th2 immune response. Accordingly, the important point here is that all samples analyzed in our study expressed the cytokines evaluated: IL-2, TNF-alpha and IFN-gamma.

There was a negative correlation between the diameter and number of fungal cells. This finding is in agreement with the literature, showing that the greater severity of the disease and proliferation of the fungi, the smaller diameter of the yeast cells (11). The number of buds and number of fungal cells were positively correlated with each other, which is a logical observation, as the number of buds depends on the number of mother cells (8). On the other hand, there was a negative correlation between bud diameter and granuloma density, which disagrees with the literature, where we find reports about the smaller size of the yeasts representing the higher activity of the disease (11) and, therefore, the lower density of the granulomas. Bud diameter and fungal cell diameter were positively correlated with each other, which agrees with reports that the size of both fungal cells and buds determines the activity of the disease (6).

There was a negative correlation between TNF-alpha and number of fungal cells and between TNF-alpha and number of buds. These results were expected, and they are in agreement with previous reported studies, according to which higher levels of this cytokine are associated with benign paracoccidioidomycosis (10). High levels of TNF-alpha are characteristic of the Th1 cellular immune response pattern observed in immune competent patients who have already been infected with *P. brasiliensis* without developing paracoccidioidomycosis. Also, during the development of the disease, the cytokine is required for granuloma formation (1).

Erythrocytes and number of fungal cells, erythrocytes and number of buds, and leukocytes and number of buds as well were positively correlated. At first, these

correlations seemed a little strange, but it is important to recall some points here. We did not classify the patients in our sample concerning rates of tobacco use because all of them were heavy smokers. Accordingly, tobacco is commonly found as a habit among chronic paracoccidioidomycosis patients and has been considered a risk factor for developing this form of the disease (4). Otherwise, the use of tobacco is related to an increase in red blood cells (erythrocytes) as a compensatory response of erythropoietin elicited by the accumulation of carbon monoxide in the body (21-23). Also, leukocytosis in tobacco smokers has been well recognized; however, its exact cause has not been elucidated (24). Studies have shown that chronic cigarette smoking exerts the release of both mature and immature polymorphonuclear neutrophils from the bone marrow resulting in increased circulating polymorphonuclear leukocytes and band cells, which contribute to the rise in leukocyte count (25). This is also associated with an increase in bone marrow turnover of polymorphonuclear leukocytes with a shortening of the mean transit time of these cells through the postmitotic pool of the marrow (26). In our results, such correlation was not observed between lymphocytes and number of buds, in agreement with the fact that leukocytosis associated with tobacco is mainly a result of neutrophilia (25,26).

In the present study, we analyzed the histological, immune and clinical features of paracoccidioidomycosis aimed at identifying their variation or correlation in patients with the adult chronic form of the disease, which, in turn, is represented by dense granulomas (27,28). However, significant differences were not found in the features analyzed, and they seemed to occur more so when comparing chronic paracoccidioidomycosis to the acute/subacute form (13). It seems that the patients with the former type of the disease do not show significant contrast when compared to each other, which suggests that they share a similar immunological picture during the

development of the disease. Although a larger sample could have provided different results, it is possible that in clinical terms, these mathematical differences would not determine prognostic changes. Moreover, our findings are corroborated by the fact that almost all patients who develop chronic paracoccidioidomycosis are males who smoke tobacco and drink alcohol excessively, which are important risk factors for the disease (29-31). Considering our results, new studies either comparing acute/subacute and chronic disseminated forms of paracoccidioidomycosis or focusing on the Th2-like pattern of cytokine production (IL-4, IL-5, IL-6 and IL-10) in the immune response should be conducted.

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Discussão Geral

4 DISCUSSÃO GERAL

A paracoccidioidomicose é uma doença sistêmica, endêmica da Amércia Latina (Ajello; Polonelli, 1985; Almeida *et al*., 2003; Ramos-e-Silva; Saraiva, 2008; Santo, 2008) que tem sido alvo de estudos dirigidos à imunologia do hospedeiro e à virulência do fungo *Paracoccidioides brasiliensis* na tentativa de elucidar sua etiopatogenia e comportamento clínico (Bava *et al*., 1991; Cano *et al*., 1998; Kashino *et al*., 2000; Neworal *et al*., 2003; Parise-Fortes *et al*., 2006; Souto *et al*., 2000). Embora as pesquisas sejam, em sua maioria, desenvolvidas em países da região endêmica, o interesse pelo conhecimento da infecção e suas peculiaridades é ou deveria ser global, já que o agente etiológico pode permanecer em latência no hospedeiro por períodos de até 60 anos (Ajello; Polonelli, 1985; Almeida *et al*., 2003). Tal fato determina que habitantes de áreas não-endêmicas possam desenvolver casos não-autóctones ou importados da doença (Ajello; Polonelli, 1985; Almeida *et al*., 2003; Ramos-e-Silva; Saraiva, 2008), que, por sua vez, tem caráter incapacitante ou letal, se não diagnosticada e tratada adequadamente (Lyon *et al*., 2009; Santo, 2008; Shikanai-Yasuda *et al*., 2006).

O Brasil é um dos países com maior número de casos de paracoccidioidomicose, sendo o Rio Grande do Sul um estado da região endêmica em que o setor agrícola constitui importante segmento da economia com muitos trabalhadores envolvidos na atividade rural (Santo, 2008). Isso colabora para o incremento do número de casos e torna o estado economicamente vulnerável à doença em função de suas consideráveis morbidade e mortalidade (Shikanai-Yasuda *et al*., 2006). O desenvolvimento de pesquisas que contribuam com o aperfeiçoamento de técnicas diagnósticas e de métodos de manejo clínico e tratamento dos pacientes, portanto, é capaz de minimizar não apenas

a morbidade e a mortalidade da enfermidade, mas também suas repercussões no contexto socioeconômico.

No Serviço de Estomatologia do Hospital São Lucas da PUCRS todos os pacientes portadores de paracoccidioidomicose atendidos entre 1977 e o término do presente estudo apresentaram a forma crônica da doença. Os pacientes eram, em sua maioria, do sexo masculino (92%), com idade entre 29 e 75 anos, tabagistas, etilistas e tinham algum tipo de ligação com o meio rural ou atividade agrícola. O fato de esses pacientes exibirem variações do comprometimento clínico pela doença, associado aos relatos da literatura de que a arquitetura histológica dos granulomas está vinculada à variação da produção de determinadas citocinas (Alves *et al*., 2009; Da Silva *et al*., 2009; Naranjo *et al*., 2010; Parise-Fortes *et al*., 2006) despertou o interesse pela presente investigação.

Algumas pesquisas têm demonstrado que a produção adequada de IFN-gama, IL-2 e TNF-alfa determina resistência à infecção ou desenvolvimento de quadros *benignos* da doença, enquanto a prevalência de IL-4, IL-5, IL-10 e intensa produção de anticorpos, com baixos níveis de IFN-gama, IL-2 e TNF-alfa associa-se à maior susceptibilidade à infecção e maior risco de desenvolvimento da forma disseminada (Livonesi *et al*., 2009; Neworal *et al*., 2003). Ao compararem-se, no presente estudo, a expressão de IFN-gama, IL-2 e TNF-alfa com o grau de compactação dos granulomas das lesões orais, não foi verificada variação significativa. Em relação à quantificação das partículas fúngicas, embora existam relatos de que seu menor diâmetro e maior quantidade estão associados à maior intensidade da infecção (Almeida *et al*., 2003; Ramos-e-Silva; Saraiva, 2008), somente o diâmetro dos brotamentos foi significativamente maior no grupo de granulomas de compactação intermediária. Também as variáveis clínicas e hematológicas não diferiram significativamente entre os grupos de diferente

compactação dos granulomas. Tais achados sugerem duas possibilidades: (a) que o tamanho da amostra seja reduzido para esse tipo de análise; ou que, (b) de fato, os pacientes que desenvolvem a forma crônica de paracoccidioidomicose não exibem diferenças significativas no que se refere à sua resposta imunológica ao *Paracoccidioides brasiliensis.* Por outro lado, é importante ressaltar que, ao investigar a produção de citocinas associadas às respostas Th1 e Th2 no soro de portadores da doença, Marques Mello *et al.* (2002) afirmam que o ponto crucial na resposta imunológica de pacientes resistentes e susceptíveis à infecção não consiste propriamente na menor produção das citocinas associadas à resposta Th1 (IL-2, IFN-gama e TNF-alfa) e sim, na superprodução das citocinas associadas à resposta Th2 (IL-4, IL-5, IL-10).

Também merece atenção a correlação negativa verificada entre a expressão imunoistoquímica de TNF-alfa e o número de fungos, bem como a correlação negativa dessa citocina com o número de brotamentos. Considerando-se que a maior intensidade de proliferação do fungo corresponde à maior intensidade da infecção (Almeida *et al*., 2003; Ramos-e-Silva; Saraiva, 2008), deduz-se que a expressão de TNF-alfa está inversamente correlacionada à intensidade da infecção. Ou seja, maiores níveis dessa citocina nas lesões de paracoccidioidomicose estarão associados à menor intensidade da doença. Achado este que é corroborado por outras pesquisas (Bava *et al*., 1991; Calich; Kashino, 1998; Kashino *et al*., 2000; Livonesi *et al.*, 2009; Parise-Fortes *et al*., 2006; Souto *et al*., 2000), embora algumas delas se refiram a dosagens de TNF-alfa no soro e não nos sítios de lesão (Benard *et al*., 2001; Fornari *et al*., 2001; Peraçoli *et al*., 2003).

Os casos de paracoccidioidomicose crônica não se associam a quadros de imunodepressão tão intensos quanto os verificados na forma aguda da doença. De modo geral, na forma crônica as alterações do sistema imunológico causadas pelo tabaco (Arnson *et al*., 2010; Sopori; Kozak, 1998) e pelo álcool (Martinez; Moya, 1992)

parecem colaborar significativamente para tornar o paciente susceptível à infecção. Já a forma aguda, ao mesmo tempo em que se expressa com comportamento clínico mais agressivo, também requer grau de imunodepressão mais intenso, existindo relatos da literatura sobre alterações críticas das citocinas envolvidas (Livonesi *et al*., 2009; Peraçoli *et al*., 2003). O presente estudo não identificou variação significativa de fatores clínicos, histomorfométricos e imunológicos em uma amostra padronizada de portadores da forma crônica de paracoccidioidomicose. Tal resultado sugere que esses pacientes, ao estarem sujeitos aos mesmos fatores de imunodepressão e risco à doença, não diferem significativamente entre si no que se refere aos aspectos avaliados.

Assim, torna-se fundamental que pesquisas futuras investiguem não apenas as citocinas pró-inflamatórias envolvidas no comportamento das lesões orais da paracoccidioidomicose crônica, mas também as anti-inflamatórias como IL-4, IL-5, IL-10 (Neworal *et al*., 2003). A inclusão da análise sérica dessas últimas no intuito de investigarem-se possíveis associações e correlações entre seu padrão de expressão e as variáveis clínicas e histológicas também pode trazer informações importantes, já que são escassos os estudos que relacionam essas duas modalidades em amostras de pacientes (Neworal *et al*., 2003; Parise-Fortes *et al*., 2006). Ainda, a inclusão de formas agudas da paracoccidioidomicose nas investigações também pode elucidar alguns aspectos obscuros da etiopatogenia e do comportamento clínico da doença.

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Anexos

ANEXO A

Submission Confirmation

Thank you for submitting your manuscript to *Mycoses*.

Manuscript ID:	MYC-RA-2012-171
Title:	Important aspects of oral paracoccidioidomycosis – a literature review
Authors:	Abreu e Silva, Mariana Salum, Fernanda Figueiredo, Maria Cherubini, Karen
Date Submitted:	06-Jul-2012

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ANEXO B

Normas para submissão de manuscritos ao periódico *Mycoses*

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ANEXO C

Journal of Oral Pathology & Medicine

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Submission Confirmation

Thank you for submitting your manuscript to *Journal of Oral Pathology and Medicine*.

Manuscript ID:	JOPM-07-12-OA-2240
Title:	Interrelationship of clinical, histomorphometric and immunohistochemical features of oral lesions in chronic paracoccidioidomycosis
Authors:	Abreu e Silva, Mariana Salum, Fernanda Figueiredo, Maria Lopes, Tiago Da Silva, Vinicius Cherubini, Karen
Date Submitted:	13-Jul-2012

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ANEXO D

Normas para submissão de manuscritos ao periódico *Journal of Oral Pathology and Medicine*

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ANEXO E

Comissão Científica e de Ética
Faculdade da Odontologia da PUCRS

Porto Alegre 15 de Dezembro de 2010

O Projeto de: Dissertação

Protocolado sob nº:	0102/10
Intitulado:	Padrão histomorfométrico e imunoistoquímico da paracoccidioidomicose oral e sua associação com fatores clínicos
Pesquisador Responsável:	Profa. Dra. Karen Cherubini
Pesquisadores Associados	Mariana Álvares de Abreu e Silva
Nível:	Dissertação / Mestrado

Foi ***aprovado*** pela Comissão Científica e de Ética da Faculdade de Odontologia da PUCRS em *15 de Dezembro de 2010.*

Este projeto deverá ser imediatamente encaminhado ao CEP/PUCRS

Profa. Dra. Ana Maria Spohr
Presidente da Comissão Científica e de Ética da
Faculdade de Odontologia da PUCRS

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ANEXO F

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMITÊ DE ÉTICA EM PESQUISA

OF.CEP-032/11 Porto Alegre, 07 de janeiro de 2011.

Senhora Pesquisadora,

O Comitê de Ética em Pesquisa da PUCRS apreciou e aprovou seu protocolo de pesquisa registro CEP 10/05316 intitulado **"Padrão histomorfométrico e imunoistoquímico da paracoccidioidomicose oral e sua associação com fatores clínicos"**.

Salientamos que seu estudo pode ser iniciado a partir desta data.

Os relatórios parciais e final deverão ser encaminhados a este CEP.

Atenciosamente,

Prof. Dr. Rodolfo Herberto Schneider
Coordenador do CEP-PUCRS

Ilma. Sra.
Profa. Karen Cherubini
FO
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