Potential Histopathological and Immune Biomarkers in Malignant and Non-Malignant Oral Lesions

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ABSTRACT

Objectives: The presented case-control study was developed to characterize the clinical, histopathological and immunological profile of patients with traumatic injuries, benign neoplasms, potentially malignant oral disorders and malignant neoplasms of the oral cavity, in order to identify biomarkers of malignancy.
Material and Methods: Clinical information was collected from dental records and several techniques were performed, including histopathological evaluation in sections stained with haematoxylin and eosin, immunohistochemistry for programmed death ligand-1 and measurement of serum levels of interferon-gamma, interleukin-6, -10 and -12. Statistical analysis was performed using IBM SPSS® Statistics software.
Results: This study included 8 patients with traumatic injuries, 8 with benign neoplasms, 6 with potentially malignant oral disorders and 11 with malignant neoplasms. An association was observed between the classification of the lesion and smoking ($P < 0.05$), the size of the lesion ($P < 0.05$), the density of the inflammatory infiltrate ($P < 0.001$), the degree of dysplasia ($P < 0.01$) and programmed death ligand-1 expression ($P < 0.01$).
Conclusions: Therefore, it is suggested that smoking, the size of the lesion, the inflammatory infiltrate and the programmed death ligand-1 expression can be considered potential biomarkers of oral malignancy.

Keywords: immune checkpoint inhibitors; mouth diseases; tumor biomarkers; tumor microenvironment.

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INTRODUCTION

The oral cavity is an anatomical site with particular physiological characteristics, being directly exposed to environmental factors, such as smoking, viral infections, food and the use of prostheses. Thus, this site is highly susceptible to a wide spectrum of injuries, including traumatic and reactive injuries, benign proliferative disorders, potentially malignant disorders and neoplasms [1].

In Brazil, oral cancer (OC) is the 5th most common cancer in men and the 13th most frequent in women. The main risk factors of OC are smoking and alcoholism. Other risk factors including excessive sun exposure (in the cases of lip cancer), obesity, human papillomavirus infection (in the case of oropharyngeal cancer) and exposure to work-related carcinogens [2].

The diagnosis of OC comprises the clinical evaluation and the performance of complementary exams. Clinical examination followed by histopathological analysis of the tissue remains the gold standard for diagnosis and therapeutic indication [3].

The identification of biomarkers has been the subject of several studies in the diagnostic and prognostic evaluation and in the management of different oral lesions, especially in malignant disorders. Tissue, serum and saliva samples are used in the evaluation of the genome, transcriptome, metabolome and proteome in studies of potential biomarkers [4].

Regarding OC, studies indicate that the concentration of proinflammatory cytokines, such as interleukin (IL) 1 beta, IL-6, IL-8, IL-12 and tumour necrosis factor can significantly reduce the proliferation of neoplastic cells [5,6]. On the other hand, an inflammatory microenvironment can favour the expression of inhibitory molecules by the cells of the tumour microenvironment, such as programmed death-1 (PD-1) and its ligand (PD-L1), in an attempt to evade the immune system, leading to a worse prognosis for patients [7,8].

In this context, studies that allow a better understanding of the immune response in oral disorders and tumour progression and its association with the tissue microenvironment are essential in the early diagnosis, prognosis and treatment approach, mainly in the indication of immunotherapy. Therefore, it is a case-control study that was developed to characterize the clinical, histopathological and immunological profile of patients with traumatic injuries, benign neoplasms, potentially malignant oral disorders and malignant neoplasms of the oral cavity, in order to identify biomarkers of malignancy.

MATERIAL AND METHODS

Selection of patients

This study included patients who visited a public dental service in Jataí, Brazil between July 2017 and February 2020 for traumatic injuries (TI), benign tumors (BN), potentially malignant oral disorders (PMOD), or malignant tumors (MN). Nine control subjects, matched for sex and age, seen at the same health service, were included. These individuals were submitted to oral cavity analysis for oral hygiene and to exclude any clinically identifiable lesions. In this study, only mucosal lesions were included. Patients that presented allergies, autoimmune diseases or systemic inflammatory conditions and/or that use corticosteroids and anti-inflammatory drugs were excluded. This study was approved by the Research Ethics Committee of Federal University of Goiás (#3,443,761).

Clinical evaluation

The patients were submitted to the collection of clinical data in standardized dental record. For this study, information was collected on age, sex, ethnicity, harmful life habits (smoking, drinking, excessive sun exposure and others) and clinical data of the lesion (anatomical site, size, shape and colour).

Sample collection

Patients with oral lesions were submitted to incisional or excisional biopsy, depending on the clinical situation. The material was stored in 10% formaldehyde. In addition, samples of venous whole blood were collected from all patients and controls. The samples were processed and stored at -20 ºC.

Histopathological analysis

Tissue samples were embedded in paraffin and sectioned in a semiautomatic microtome (RM2235 - Leica Microsystems; Wetzlar, Germany) in consecutive cuts of 4 µm thick. The materials were submitted to histological processing and stained with haematoxylin and eosin, according to protocol described in the literature [9].

For histological gradation, PMOD were classified according to the degree of dysplasia (mild, moderate and severe) [10] and MN were classified according to differentiation (well, moderately and poorly) [11]. For all lesions, the inflammatory infiltrate was classified in the cross system (+) as: 3+ (frequent

and very intense), 2+ (frequent and intense), 1+ (occasional and attenuated), ± (rare and weak) and 0 (absent) [12]. In MN, analysis of tumour infiltrating lymphocytes (TILs) was also included, according to the protocol adapted by our research group, which are classified in 0 to 10%, 11 to 40% and 41 to 90% [13]. The analyses were performed by two independent pathologists, including a medical and an oral pathologist.

Immunohistochemical analysis

Two 4 μm sections, serialized from the material obtained for haematoxylin and eosin staining, were assembled in the analysis signalled by the marking of the PD-L1 molecule. Anti-PD-L1 antibody (clone CAL10, 1 : 400 titulation - Biocare Medical, LLC; Pacheco, California, USA) and the Starr Trek Universal HRP Detection System Kit (Biocare Medical, LLC; Pacheco, California, USA) were used. A sample of human tonsil tissue was used as positive control. Two slides from each case were used, the negative control and the case itself.

The material was deparaffinized in xylol and hydrated in baths of decreasing alcohol. The formolic pigment was removed with 24% ammonium hydroxide. The protocol was completed according to internal and manufacturer recommendations, including antigenic recovery in an autoclave cycle, endogenous peroxidase block with 3% hydrogen peroxide, endogenous protein block, overnight incubation with primary antibody, incubation with secondary antibody labelled with horseradish streptavidin (HRP) and stained with 3,3'-diaminobenzidine (DAB). The slides were counterstained with Harris' haematoxylin.

The determination of the frequency of PD-L1+ cells and the staining profile (membrane or cytoplasm) considered positive staining for greater than or equal (≥) to 50% in tumour cells or ≥ 1% for combined staining in tumour cells and immune cells [14]. Cases that showed expression only in immune cells were also evaluated. PD-L1 expression was also stratified as 1%, 1 to 5%, 5 to 20% and > 20%, according to the previous determination of our study group [13].

Serum cytokine determination

The enzyme-linked immunosorbent assay (ELISA) reaction was performed using a sandwich antigen principle, according to manufacturer's specifications, including sensitization of the plate with capture antibody, elimination of the background, determination of the standard curve, incubation of the sample, addition of detection antibody, streptavidin-HRP complex and chromogen and reading of the optical density in a spectrophotometer at 450 nm (Celer/Polaris® - Celer Biotechnology S/A; Belo Horizonte, Minas Gerais, Brazil). ELISA kits were used to detect serum IL-6, IL-10, IL-12 and interferon gamma (IFN-γ) cytokines (Uncoated ELISA - Invitrogen™; Waltham, Massachusetts, USA).

Statistical analysis

Statistical analysis was performed using IBM SPSS® Statistics software version 26.0.0.0 (IBM Corp.; Armonk, New York, USA). Association tests were used, including the Chi-square (χ^2) test and the Kruskall Wallis test. Spearman's correlation (ρ) was also done. The numeric correlation values were categorized as very weak (ρ = 0 to 0.19), weak (ρ = 0.2 to 0.39), moderate (ρ = 0.4 to 0.69), strong (ρ = 0.7 to 0.89) and very strong (ρ = 0.9 to 1) [15]. The data were presented as mean and standard deviation (M [SD]). The level of significance adopted for all analyses was $P < 0.05$.

RESULTS

This study included 8 patients with BN, 6 with PMOD, 11 with MN, 8 with TI and 9 patients without injury. Epidemiological and clinical variables are summarized in Table 1.

Gender, age and ethnicity did not present significant difference between the groups. A significant association were found between smoking and injury classification ($\chi^2 = 14.491$; $P < 0.05$), being majority of individuals with malignant neoplasms smokers or ex-smokers (n = 9; 81.8%). Alcohol use, sun exposure, sedentary lifestyle and illicit drug use were not associated with injury classification.

Concerning the lesion site, no significant association was found. On the other hand, the size lesion was significant associated with injury classification ($\chi^2 = 8.774$; $P < 0.05$), being the size ≥ 2 cm present mainly in MN.

The diagnosis of BN included cases of papilloma (n = 3; 37.5%), haemangioma (n = 3; 37.5%), nevus (n = 2; 25%). PMOD patients presented lesions compatible with actinic cheilitis (n = 3; 50%), leukoplakia (hyperkeratosis and acanthosis with areas of dysplasia; n = 2; 33.3%) and scleroatrophic lichen planus (n = 1; 16.7%). Most MN were compatible with oral squamous cell carcinoma (OSCC) (n = 9; 81.8%), but two cases were not conclusive as to the histological subtype, being diagnosed as malignant

Table 1. Epidemiological and clinical parameters of cases (n = 42)

Variables	Without injury (n = 9)	Traumatic injury (n = 8)	Benign neoplasm (n = 8)	Potential malignant oral disease (n = 6)	Malignant neoplasm (n = 11)	P-value
	N (%)	N (%)	N (%)	N (%)	N (%)	
Gender						
Male	6 (66.7)	5 (62.5)	3 (37.5)	3 (50)	9 (81.8)	0.365[b]
Female	3 (33.3)	3 (37.5)	5 (62.5)	3 (50)	2 (18.2)	
Age (years old), mean (SD)	55.1 (SD 1.1)	50.5 (SD 2.3)	53.1 (SD 8.4)	57.8 (SD 3.2)	64.4 (SD 1.2)	0.433[c]
Ethnicity						
White	6 (66.7)	2 (25)	2 (25)	5 (83.3)	6 (54.5)	0.198[b]
Black	3 (33.3)	6 (75)	6 (75)	1 (16.7)	5 (45.5)	
Harmful habits						
Smoker/ex-smoker	2 (22.2)	4 (50)	2 (25)	2 (33.3)	9 (81.8)	0.022[a]
Alcohol user/ex-alcoholic	1 (11.1)	3 (37.5)	3 (37.5)	1 (16.7)	4 (36.4)	0.097[b]
Sun exposure	0 (0)	0 (0)	0 (0)	2 (33.3)	3 (27.3)	0.316[b]
Sedentary lifestyle	0 (0)	1 (12.5)	2 (25)	0 (0)	3 (27.3)	0.369[b]
Illicit drug use	0 (0)	0 (0)	0 (0)	1 (16.7)	1 (9.1)	0.41[b]
Lesion site						
Tongue	NA	2 (25)	2 (25)	0 (0)	4 (36.4)	0.23[b]
Lower lip	NA	1 (12.5)	3 (37.5)	3 (50)	2 (18.2)	
Throughout the oral mucosa	NA	0 (0)	0 (0)	1 (16.7)	0 (0)	
Palate	NA	1 (12.5)	1 (12.5)	0 (0)	1 (9.1)	
Gingival mucosa	NA	0 (0)	1 (12.5)	0 (0)	0 (0)	
Alveolar ridge	NA	0 (0)	1 (12.5)	2 (33.3)	2 (18.2)	
Buccal mucosa	NA	4 (50)	0 (0)	0 (0)	2 (18.2)	
Size of the lesion						
< 2 cm	NA	7 (87.5)	7 (87.5)	4 (66.7)	2 (18.2)	0.032[a]
≥ 2 cm	NA	1 (12.5)	1 (12.5)	2 (33.3)	9 (81.8)	

[a]Statistically significant at level P < 0.05 (Chi-square test).
[b]No statistically significant at level P < 0.05 (Chi-square test).
[c]No statistically significant at level P < 0.05 (Kruskall Wallis test).
N = number; NA = not applied; SD = standard deviation.

epithelial neoplasm (n = 2; 18.2%). Finally, patients with TI presented a predominance of fibroma (n = 7; 87.5%) and one of the patients (n = 1; 12.5%) presented epithelial proliferative changes without dysplasia.

Table 2 summarizes the histopathological and immunological data. The inflammatory infiltrate was rare (n = 4; 50%) in BN, mild in PMOD (n = 5; 83.3%) and in TI (n = 8; 100%) and intense in MN (n = 5; 45.5%), with a significant association between the degree of inflammatory infiltrate and the classification of the lesion (χ^2 = 32.79; P = 0.001). The evaluation of TILs revealed a majority of cases of NM with an infiltration between 11 and 40% (n = 7; 63.6%), followed by 0 to 10% (n = 3; 27.3%) and 41 to 90% (n = 1; 9.1%).

Regarding structural changes, there was a predominance of cases without dysplasia in BN (n = 8; 100%) and TI (n = 8; 100%) and mild dysplasia in PMOD (n = 4; 66.7%), with a significant association between the degree of dysplasia and the classification of the lesion (χ^2 = 23.867; P = 0.002). As for MN, there was a predominance of moderately differentiated tumours (n = 5; 45.5%), followed by well differentiated tumours (n = 3; 27.3%) and poor differentiated tumour (n = 3; 27.3%).

PD-L1 expression was observed in 1 case of PMOD (n = 1, 16.7%) (Figure 1A), with a gradation of 1%, and in 7 cases of MN (Figure 1B), with the majority of cases ranging from 1 to 5% (n = 4, 36.4%). A significant association was found between the expression of PD-L1 and the classification of the lesion (χ^2 = 22.04; P = 0.009). PD-L1 expression was also positively correlated with the degree of

Table 2. Histopathological and immunological parameters of cases (n = 42)

Variables	Without injury (n = 9)	Traumatic injury (n = 8)	Benign neoplasm (n = 8)	Potential malignant oral disease (n = 6)	Malignant neoplasm (n = 11)	P-value
	N (%)	N (%)	N (%)	N (%)	N (%)	
Inflammatory infiltrate						
Absent	NA	0 (0)	1 (12.5)	0 (0)	0 (0)	0.001[a]
Rare	NA	0 (0)	4 (50)	0 (0)	0 (0)	
Mild	NA	8 (100)	3 (37.5)	5 (83.3)	4 (36.4)	
Moderate	NA	0 (0)	0 (0)	1 (16.7)	2 (18.2)	
Intense	NA	0 (0)	0 (0)	0 (0)	5 (45.5)	
Dysplasia						
Absent	NA	8 (100)	8 (100)	0 (0)	NA	0.001[a]
Mild	NA	0 (0)	0 (0)	4 (66.7)	NA	
Moderate	NA	0 (0)	0 (0)	2 (33.3)	NA	
PD-L1 expression						
0%	NA	8 (100)	8 (100)	5 (83.3)	4 (36.4)	0.009[a]
1%	NA	0 (0)	0 (0)	1 (16.7)	0 (0)	
1 to 5%	NA	0 (0)	0 (0)	0 (0)	4 (36.4)	
5 to 20%	NA	0 (0)	0 (0)	0 (0)	3 (27.3)	
IL-6 levels (pg/mL), mean (SD)	0.5 (SD 0.1)	0.5 (SD 1.2)	0 (SD 0)	0 (SD 0)	2.2 (SD 3.9)	0.411[b]
IL-10 levels (pg/mL), mean (SD)	3.7 (SD 11.2)	0 (SD 0)	1.5 (SD 3.6)	0 (SD 0)	0.4 (SD 1.1)	0.44[b]
IL-12 levels (pg/mL), mean (SD)	0 (SD 0)	0 (SD 0)	0.3 (SD 0.6)	0 (SD 0)	0 (SD 0)	0.305[b]
IFN-γ levels (pg/mL), mean (SD)	0 (SD 0)	0 (SD 0)	1.1 (SD 3)	0 (SD 0)	15.4 (SD 40.6)	0.496[b]

[a]Statistically significant at level $P < 0.05$ (Chi-square test).
[b]No statistically significant at level $P < 0.05$ (Kruskall Wallis test).
N = number; NA = not applied; SD = standard deviation; PD-L1 = programmed death ligand-1; IL = interleukin; IFN-γ = interferon gamma.

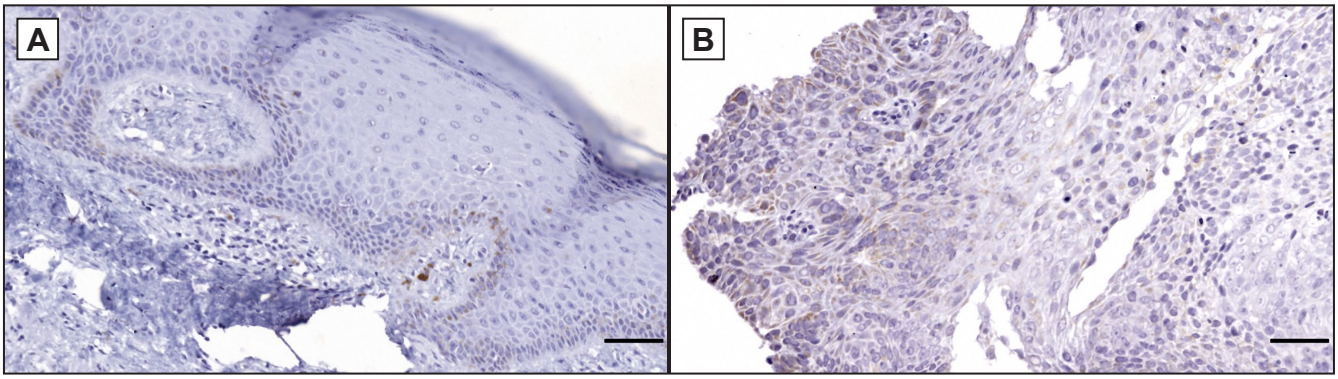

Figure 1. Immunohistochemistry for programmed death ligand-1 (PD-L1). Scale bar: 50μm. Original magnification x200.
A = expression of PD-L1 in a case of actinic cheilitis. The expression is located mainly in the basal epithelial cells and in some inflammatory cells. B = greater expression of PD-L1 in a case of oral squamous cell carcinoma. Several tumour cells and some immune cells showed the expression of the molecule.

inflammatory infiltrate ($\rho = 0.63$; $P < 0.001$) and with the density of TILs ($\rho = 0.588$; $P < 0.05$). However, PD-L1 expression was not correlated with dysplasia, gradation and serum cytokine levels ($P > 0.05$).

The differences between cytokine serum levels were not statistically significant. However, we highlight that the serum IL-10 levels increased in the control group compared to other groups, while IFN- γ was higher in MN when compared to other groups. Serum levels of IFN-γ were correlated only with the levels of IL-6 ($\rho = 0.462$; $P < 0.05$) and IL-12 ($\rho = 0.676$; $P < 0.001$). The serum levels of cytokines were not correlated with epidemiological and clinical parameters ($P > 0.05$).

DISCUSSION

The literature suggests that male individuals have a greater predilection for the development of malignant neoplasms, as observed in our study, especially due to the higher prevalence of smoking and alcohol consumption in this population [16]. In addition, it is suggested that women are at increased risk of developing traumatic and/or vascular and connective tissue injuries, possibly due to hormonal conditions [1]. This observation applies to our study in the context of benign neoplasms, but not in the context of traumatic injuries. Besides that, the prevalence of oral lesions is higher in individuals over 45 years old, as observed in this study, mainly due to the accumulation of harmful habits and iatrogenic factors in the oral cavity [1]. There is a progressive increase in the incidence of oral carcinoma in older individuals, as observed in our study, reaching a plateau around 70 to 79 years [16].

Smoking and alcohol consumption are important risk factors in the progression of oral lesions, acting in isolation or synergistically. Both conditions alter the metabolism of the cells of the oral mucosa and facilitate and/or promote genetic mutations [16]. Thus, this study reinforces the importance of these risk factors in the context of oral lesions, especially in PMOD and MN.

The labial mucosa and the buccal vestibule are more exposed to deleterious factors such as chronic irritation, mechanical and thermal injuries and bites [1]. That characteristic justifies the susceptibility of these regions to oral lesions, as observed in this study, which showed a predominance of BN, TI and PMOD in these regions. In the case of oral carcinoma, tongue lesions predominate, especially in the lateral border [17], as observed in this study.

Oral MN can appear as spots, plaques, tumours or ulcers, erythematous, leukoplastic, necrotic or mixed in colour, with a size greater than two centimetres [3,17]. Benign lesions, on the other hand, usually manifest as pedunculated or sessile erythematous mass with less than one centimetre in size [1]. These findings agree with what was observed in this study, being suggested that size may assist in the differential diagnosis, considering the greater frequency of MN greater than two centimetres.

The inflammatory infiltrate is an essential component of the tumour microenvironment. Recent studies demonstrate that the infiltrate can contribute to tumour progression, stimulation tissue invasion, or in its control, through the recognition and destruction of tumour cells. In terms of immunofluorescence assay, there was a general increase in the inflammatory infiltrate of patients with PMOD, compared to individuals with non-neoplastic lesions, and patients with OSCC presented a greater infiltration in related to the other two groups [18], corroborating the histopathological findings in this study.

The assessment of the degree of dysplasia remains one of the most important parameters in the prognostic assessment of patients. It is expected that patients with PMOD have degrees of dysplasia than patients with BN and TI, considering the higher frequency of exposure to carcinogens that promote genetic and morphological changes [1,19].

The PD-1/PD-L1 axis is an important immune system inhibitor pathway with already known repercussions on tumour progression and metastasis [7]. The infiltrate of TILs, the production of cytokines and the expression of PD-L1 have already been proposed as potential biomarkers for the use of immunotherapy [14,20].

The expression of PD-L1 in dysplastic oral lesions has been described in recent studies. An increase in the expression of PD-L1 was observed in dysplastic basal cells and inflammatory cells in lesions that evolve to malignancy, indicating that the increase in the expression of PD-L1 precedes tissue invasion and malignant transformation [21,13]. This study reinforces these results, with a greater expression of PD-L1 in MN compared to PMOD, BN and TI.

The inflammatory infiltrate directly correlates with the expression of PD-L1 due to the ability of IFN-γ to induce the expression of the molecule. Thus, this study corroborates some works in the literature that indicated greater expression of PD-L1 in tumours with greater inflammatory infiltrate, especially in the case of TILs [13,22,23]. However, in this study the expression of PD-L1 did not correlate with the serum levels of IFN-γ, indicating a possible divergence between the tissue and serum microenvironment.

IL-6 has different biological activities, including increasing the release of transcription factors such as AP-2, capable of activating the Ras/MAPK pathway, in addition to inactivating the p53 gene, suppressing apoptosis and promoting progressive cell growth. There are already reports in the literature that higher levels of salivary IL-6 are detected in patients with oral cancer compared to patients with leukoplakia, chronic inflammatory diseases and healthy individuals, but there was no significant difference in serum IL-6 [5,6]. In this sense, it is also important to note that in this study the levels of cytokines were not correlated with comorbidities and harmful habits, which could acts as confounded factors in the analysis.

The immune response can be synthesized in two large

groups: proinflammatory response, associated with Th1 lymphocytes and cytokines such as IFN-γ and IL-12, and anti-inflammatory response, associated with Th2 lymphocytes and cytokines such as IL-10. The studies presented in the literature reveal a heterogeneity regarding the levels of cytokines in patients with head and neck cancer, reinforcing the very heterogeneity of these tumours [24,25]. In this study, a pattern of greater expression of proinflammatory cytokines, especially IFN-γ was observed in patients with malignant neoplasms, while IL-10 was more expressed in health patients.

In addition, a correlation was observed between the levels of IFN-γ, IL-12 and IL-6. These results suggest that the sample of this study included patients with inflammatory microenvironments, mainly in malignant lesions, despite the lack of statistical significance between the histopathological evaluation and cytokine measurement. Thus, it is expected that these patients are potential candidates for immunotherapeutic treatments and will evolve with a good prognosis.

It is also important to note that the detection of cytokines in unstimulated serum is hardier than in stimulated samples [26], which could justify the elevate number of samples with no detection of cytokines in this study. So, it is possible that in some cases, such as OPMD diseases, the lack of detection indicate a false negative result.

CONCLUSIONS

This study presented the limitations of the study sample, the heterogeneity of lesions and the lack of detection of cytokines in several unstimulated samples. Despite of that, this study endorses the importance of clinical, histopathological and immunological evaluation in the early diagnosis and prognosis of patients with oral lesions, besides the possibility of individualization in the treatment of the patient. It is suggested the importance of potential biomarkers in the identification of malignant lesions, especially smoking, lesions larger than > 2 centimetres, more intense inflammatory infiltrate and greater expression of programmed death ligand-1. In addition, the identification of inflammatory microenvironments associated with greater expression of programmed death ligand-1 may be relevant in the context of the indication and prognosis of immunotherapeutic treatment. It is also highlighted that new studies should be developed in order to minimize the confounded factors in microenvironment analysis of oral lesions and to identify histopathological and immune biomarkers of prognosis.

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