Is salivary epidermal growth factor a biomarker for oral leukoplakia? A preliminary study

Filipe Jaeger, DDS, MSc, PhD student,[a] Ana Carla Assunção, DDS,[a]
Patrícia Carlos Caldeira, DDS, MSc, PhD, Post-doctoral researcher,[a]
Celso Martins Queiroz-Junior, DDS, MSc, PhD, Post-doctoral researcher,[a]
Vanessa Fátima Bernardes, DDS, MSc, PhD, Professor,[b] and
Maria Cássia Ferreira de Aguiar, DDS, MSc, PhD, Professor[a]

Objectives. The aim of this study was to compare the salivary epidermal growth factor (EGF) levels between patients with oral leukoplakia (OL) and clinically healthy individuals, to evaluate the association between salivary and tissular EGF, and to correlate EGF with clinicopathologic data, including the presence of dysplasia.
Study Design. Salivary EGF levels were measured in 32 patients and 32 controls. The tissue expressions of EGF and its receptor (EGFR) were immunohistochemically evaluated.
Results. Salivary EGF levels were similar in patients with OL compared with controls. There was no association between the salivary levels and immunohistochemical expression of EGF. An absence of EGF detection by immunohistochemistry was associated with development of multiple lesions. Dysplastic lesions showed a tendency toward presenting higher salivary EGF levels.
Conclusions. Currently, it is not possible to indicate salivary EGF as a biomarker for OL. Further studies are needed to elucidate the role of EGF in oral carcinogenesis. A follow-up study is necessary to evaluate the changes in EGF values following the surgical excision of OL. (Oral Surg Oral Med Oral Pathol Oral Radiol 2015;119:451-458)

Oral leukoplakia (OL), a potentially malignant disorder, is defined as a white plaque of questionable risk that cannot be clinically or pathologically characterized as any other disease.[1] The rates of malignant transformation are variable and are influenced by gender, the clinical aspects of OL, association with tobacco use, and the presence of epithelial dysplasia.[2,3] Evidence suggests that tobacco and alcohol use may be associated with at least a subset of OL cases;[4] however, this evidence is primarily based on the results of observational studies that report the regression of OL following cessation of tobacco use.[5] There is a lack of reliable markers associated with the pathogenesis of OL.

Epidermal growth factor (EGF), a 53-amino acid polypeptide, was originally isolated from mouse salivary glands. In humans, the major sources of EGF are the parotid glands and kidneys.[6] EGF is a member of a family of peptide growth factors that activate EGF receptors. EGF/EGFR signaling pathway plays important roles in the proliferation, differentiation, and migration of a

variety of cells, particularly epithelial cells.[7] Therefore, as a mitogen, EGF can function both in the maintenance of epithelial integrity[6] and in carcinogenesis.[8] The EGF/EGFR signaling pathway has been implicated in the pathogenesis of a variety of tumors, including oral squamous cell carcinoma.[9] A recent study suggested that EGFR represents a sensitive marker to identify the neoplastic potential of neoplastic tissues.[10]

The immunohistochemical expression of EGF has been correlated with tumor progression in the oral mucosa.[11] Some studies have reported lower levels of EGF in the saliva of patients who smoke[12,13] compared with nonsmokers. In previous studies from our research group, reduced salivary levels of EGF were identified in patients with oral squamous cell carcinoma (OSCC), and this reduction was influenced by smoking, tumor location, and alcohol consumption.[13,14] Thus, if tobacco use diminishes the EGF concentration in saliva, the EGF pathway might be involved in oral carcinogenesis.

On the basis of these considerations, we hypothesized that salivary EGF could also be reduced in OL, thereby

Statement of Clinical Relevance

This pilot study evaluated whether salivary epidermal growth factor (EGF) could be used as a biomarker for oral leukoplakia (OL); however, there was no difference in the levels of EGF in the saliva of patients and healthy controls.

potentially participating in the early stages of oral carcinogenesis. Therefore, the aims of this study were to verify the salivary levels of EGF in patients with OL and to compare these levels with those of healthy individuals. Furthermore, clinicopathologic features that are considered risk factors for the malignant transformation of OL, including the presence or absence of dysplasia, were compared with the salivary levels and immunoexpression of EGF and EGFR in tissues.

MATERIALS AND METHODS
Patients
The study protocol was approved by the Ethics Committee of the Federal University of Minas Gerais (07330712.5.0000.5149), and all participants signed an informed consent form. The study was conducted in compliance with the Helsinki Declaration.

Patients with OL who attended the Oral Medicine Clinics of the School of Dentistry of the Federal University of Minas Gerais were enrolled in the study (case group). The case group included patients with newly diagnosed OL lesions as well as patients who were under follow-up for previously diagnosed OL and still presented an OL lesion. Clinical data, including age, gender, the number and location of lesions, and smoking and alcohol habits, were recorded during the clinical examination. Individuals included in the control group were healthy, had no oral lesions, and were matched with the case group by gender, age, and smoking habits.[15] The amount of cigarettes used were considered for matching. In accordance with our previous studies, individuals were not matched for alcohol consumption because of the difficulty in matching controls with the same or similar alcohol consumption profiles. Patients and controls with signs of significant medical problems, such as congestive heart failure, active infection, autoimmune disease, hepatitis, human immunodeficiency virus (HIV) infection, or abnormal renal function were excluded from the study.[14] The clinical information was correlated with salivary EGF levels.

Saliva and OL tissue samples
Patients refrained from eating, drinking, smoking, and performing oral hygiene procedures for at least 1 hour before saliva collection. A cotton wool swab (Salivette, Sarstedt AG and Co., Numbrecht, Oberbergischer Kreis, Germany) was placed in the mouth for 5 minutes. The samples were immediately diluted (1:1) in phosphate buffered saline solution that contained protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzethonium chloride, 10 mM ethylenediaminetetraacetic acid, and 0.01 mg/mL aprotinin A) and 0.05% Tween-20. The processed samples were

stored at $-20°C$ for use in enzyme-linked immunosorbent assay (ELISA).

Hematoxylin-eosin stained sections of formalin-fixed, paraffin-embedded materials from incisional or excisional lesion biopsies were evaluated and used for immunohistochemistry. Twenty-six OL samples were selected and histopathologically classified into two groups according to the presence or absence of epithelial dysplasia, on the basis of the WHO recommendation.[2] The tongue and the floor of the mouth were considered high-risk sites, whereas all other sites were considered low risk.[3]

Enzyme-linked immunosorbent assay
Salivary EGF levels were measured via a sandwich ELISA by using a commercially available kit, Human EGF (DuoSet, R and D Systems, Minneapolis, MN), in accordance with the manufacturer's instructions. The total protein content in the saliva was determined using the Bradford method[16] (Sigma, St. Louis, MO); bovine serum albumin was used as the standard (Fermentas Life Sciences, Vilnius, Lithuania). The total protein content was used to normalize the EGF values for each sample.

To investigate the relationship between salivary EGF levels and categorical variables examined, the median EGF value in the saliva of the OL patients was established (= 0.046). The median was used as the cutoff value to categorize EGF as "low" if the value was equal to or lower than the median or "high" if it was higher than the median.

Immunohistochemical staining
First, 3-μm sections were deparaffinized in xylenes and hydrated with graded ethanol. Following endogenous peroxidase blockage, antigen retrieval was performed with TRIS-EDTA solution, pH 8.0. The specimens were then incubated with anti-EGF (1:50) and anti-EGFR (1:100) antibodies (clone EGF-10 [Santa Cruz Biotechnology, Inc., Santa Cruz, CA], and clone 31 G7 [Zymed Laboratories Inc., San Francisco, CA], respectively). Dako System Advance HRP was then applied (Dako, Carpinteria, CA, code-k4068). The reaction was revealed with DAB chromogenic solution and counterstained with Mayer hematoxylin. Negative controls were obtained by omitting the primary antibody, and samples of healthy oral mucosa of known immunoreactivity were included as positive controls (eg, retromolar mucosa from surgical excision of included third molars).

Immunohistochemical evaluation
Immunohistochemical staining was analyzed by two observers (MCFA and FJ) who were blinded to the data.

The evaluation was performed simultaneously in a two-observer microscope, and evaluation discrepancies were resolved by discussing the cases. The expression of EGF was evaluated by the extent and intensity of immunolabeling in the cytoplasm.[17] The staining intensity was classified into five groups: 0 = no staining; 1 = weak; 2 = moderate; 3 = strong; and 4 = very strong. The extent of staining was classified as follows: 0 = no immunostaining; 1 = less than 25% of epithelial cells demonstrated positivity; 2 = 25% to 50% of cells demonstrated positivity; 3 = 50% to 75% of cells demonstrated positivity; and 4 = greater than 75% of cells demonstrated positivity. A final score was established by adding the two indexes. According to this final score, the cases were divided into three groups: Negative (−) = score 0; positive (+) = scores 1 to 4; and strong positive (++) = scores 5 to 8.

For EGFR evaluation, the extent and intensity of immunolabeling in the epithelial cell membranes were classified according to a four-point scale:[18] 0 (no labeling or labeling in <10% of the cells); 1 (weak labeling, homogeneous or patchy, in >10% of the cells); 2 (moderate labeling, homogeneous or patchy, in >10% of the cells); and 3 (intense labeling, homogenous or patchy, in >10% of the cells). These scores were then grouped into two categories: negative (0 and 1) and positive (2 and 3).

Statistical analysis

The statistical analysis was performed with the use of SPSS software, version 21 (SPSS Inc., Chicago, IL). The results are expressed as the mean ± standard deviation. A Shapiro-Wilk test indicated no normality for EGF ($P < .05$). The Wilcoxon test was used to compare the salivary EGF levels between the controls and the cases. A one-sample test of proportions was used to make inferences regarding the proportion in each category. The differences in categorical variables and immunoexpression of EGF and EGFR were analyzed by using the Chi-square and Fisher's exact tests, when applicable. P values less than .05 were considered significant.

RESULTS

Notably, the proportion of patients who smoked (43.8%) was similar to the proportion of patients who consumed alcohol (46.9%), and this profile was also verified for the controls (53.1% of the patients drank some alcohol).

Patient information and the clinicopathologic features of OL are shown in Table I. Most lesions were homogeneous, classified as absent dysplasia, and located in regions of low risk for malignant transformation ($P < .05$). Low-risk regions included the alveolar ridge (n = 9), the jugal (cheek) mucosa (n = 9), the retromolar

Table I. Clinicopathologic features of the oral leukoplakia group

	N (%)	P value
Gender		>.05
Female	14 (43.8)	
Male	18 (56.2)	
Age (years)		>.05
≤60	14 (43.8)	
>60	18 (56.2)	
Site*		<.05
High risk	06 (18.8)	
Low risk	26 (81.2)	
Aspects		<.05
Homogeneous	25 (78.1)	
Heterogeneous	07 (21.9)	
Number of lesions		>.05
Multiple	21 (65.5)	
Single	11 (34.4)	
Size		>.05
≤5 mm	05 (15.6)	
06−10 mm	14 (43.8)	
10−15 mm	01 (03.1)	
≥20 mm	12 (37.5)	
Smoking habit		>.05
No	18 (56.2)	
Yes	14 (43.8)	
Alcohol consumption		>.05
No	17 (53.1)	
Yes	15 (46.9)	
Epithelial dysplasia†		<.05
Present	07 (21.9)	
Absent	25 (78.1)	

*High risk, tongue and/or floor mouth; low risk, other sites.
†Present, high-risk; absent, low-risk.

region (n = 3), the alveolar mucosa (n = 2), and the hard palate, gingiva, and labial mucosa (1 case each). High-risk lesions were from the tongue (n = 6). Soft palate and lip lesions were not included because of different etiologic factors.

Salivary levels of EGF

There was no significant difference ($P = .254$) in the salivary EGF levels between the patients (0.059 ± 0.033 pg/mg protein) and the controls (0.077 ± 0.061 pg/mg protein) (Figure 1). No significant association was identified between the salivary levels of EGF and the clinical features examined (Table II). There was no association between the salivary levels of EGF and the immunohistochemical expression of EGF or EGFR.

Immunohistochemical expression of EGF

EGF was positive in 8 (30.8%) and negative in 18 (69.2%) OL samples (Table III). No sample presented a "strong positive" score. The pattern of the immunoexpression was a brown staining in the cytoplasm (Figure 2). Nuclear EGF expression was observed in 11 (42.3%)
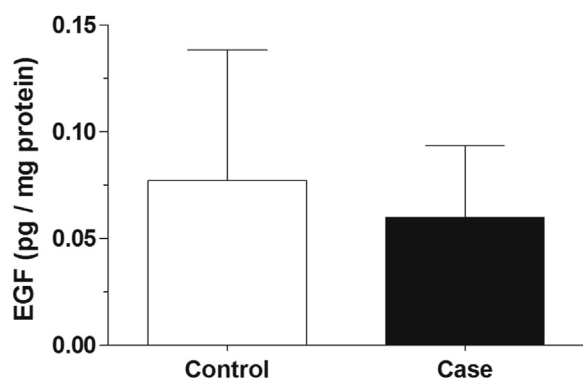
Fig. 1. Levels of epidermal growth factor (EGF) in saliva of patients with oral leukoplakia (case) and healthy patients (control), expressed as mean ± standard deviation. There was no significant difference in the EGF salivary levels between case (0.059 ± 0.033 pg/mg protein) and control groups (0.077 ± 0.061 pg/mg protein).

Table II. Association of clinicopathologic features and salivary levels of EGF

	High EGF, N (%)	Low EGF, N (%)	P
Gender			
Female	8 (57.1)	6 (42.9)	.476[*]
Male	8 (44.4)	10 (55.6)	
Age (years)			
≤60	6 (42.9)	8 (57.1)	.476[*]
>60	10 (55.6)	8 (44.4)	
Site			
High risk	2 (33.3)	4 (66.7)	.654[†]
Low risk	14 (53.8)	12 (46.2)	
Appearance			
Homogeneous	12 (48.0)	13 (52.0)	1.000[†]
Heterogeneous	4 (57.1)	3 (42.9)	
Number of lesions			
Multiple	10 (47.6)	11 (52.4)	.710[*]
Single	6 (54.5)	5 (45.5)	
Smoking habit			
No	11 (61.1)	7 (38.9)	.154[*]
Yes	5 (35.7)	9 (64.3)	
Alcohol consumption			
No	10 (58.8)	7 (41.2)	.288[*]
Yes	6 (40.0)	9 (60.0)	
Epithelial dysplasia			
Present	6 (85.7)	1 (14.3)	.083[†]
Absent	10 (40.0)	15 (60.0)	

EGF, epidermal growth factor.
*Chi-square test
[†]Fisher's exact test

OL cases, although it was not considered for immunohistochemical evaluation. There was no significant association between the immunoexpression and salivary levels of EGF (*P* = .683).

A significant association between the number of lesions and the immunoexpression of EGF was identified (Table IV). Most patients (75%) who presented with a single lesion had positive staining for EGF, whereas patients who had multiple lesions (88.9%) had negative immunoexpression for EGF (*P* = .003).

There was no significant association between EGF immunoexpression and other clinicopathologic features (Table IV).

Immunohistochemical expression of EGFR
Immunoreactivity of EGFR appeared as a brownish color in the cytoplasmic membrane of cells and was detected in 16 (61.5%) cases (Figure 3). Staining was negative in 38.5% (n = 10) of the cases. There was no association between EGFR immunoreactivity and the clinicopathologic variables examined in this study (*P* > .05).

Histopathologic features
Most OL lesions (78.1%) presented no dysplasia on histopathologic assessment. We could not find any association between salivary levels of EGF and the presence of dysplasia, although there was a tendency for patients with dysplasia to have high salivary EGF (*P* = .083). Moreover, the presence of dysplasia was not associated with EGF or EGFR immunoexpression (*P* > .05).

DISCUSSION
This study investigated salivary EGF levels and the expression of EGF and EGFR in tissues obtained from patients with OL compared with those from healthy individuals. EGF is a potent mitogen in cells that express its receptor, EGFR.[8] Although EGF plays an important role in tissue development and homeostasis, it is also overexpressed in a variety of cancer types and promotes tumorigenesis and metastasis.[19] Interestingly, the ligands of EGFR may only fully activate the cognate receptors as soluble factors because ligand-induced conformational changes are required for receptor dimerization and the generation of intracellular signals.[20] To date, no study has investigated the association between salivary EGF levels and OL.

EGF exhibits different expression levels when detected in varied biologic and pathologic states;[21] however, no study has established the normal levels of salivary EGF in healthy individuals. For this reason, median values were used in the present and previous studies[13] to categorize the values as "low" or "high."

Because this was the first study to explore salivary EGF in patients with OL, calculation of an appropriate sample size was not feasible. Alternatively, we performed a sample size calculation on the basis of the prevalence of OL in the population (2%; from a minimum sample of 28 cases).

Table III. Salivary levels and immunoexpression of EGF

	Negative immunostaining, N (%)	Positive immunostaining, N (%)	Total, N (%)
High salivary EGF	9 (75.0)	3 (25.0)	12 (46.2)
Low salivary EGF	9 (64.3)	5 (35.7)	14 (53.8)
Total	18 (69.2)	8 (30.8)	26 (100.0)

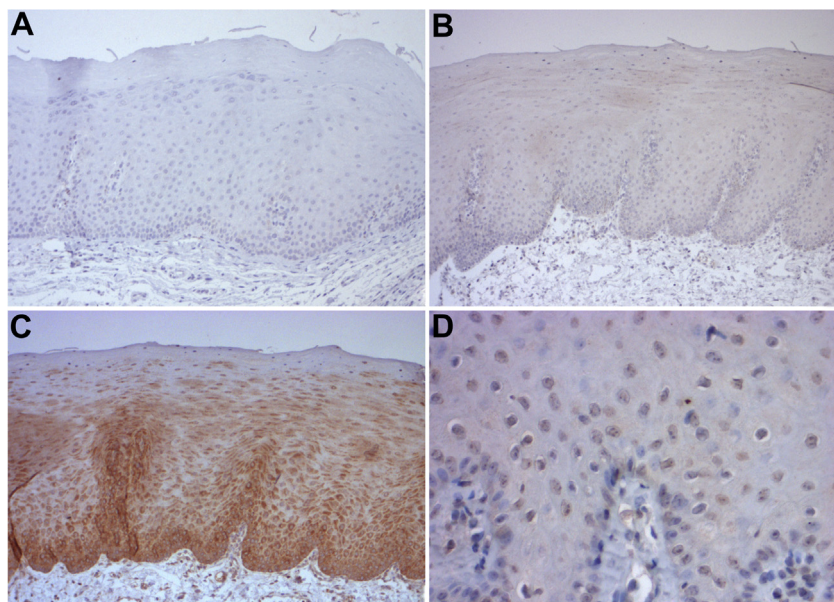*EGF*, epidermal growth factor.

Fig. 2. Epidermal growth factor (EGF) expression in oral leukoplakia (OL). **A,** OL from tongue showing hyperkeratosis and no epithelial dysplasia. This sample was negative for EGF (score 0, no staining). **B,** OL from tongue, with hyperkeratosis and mild epithelial dysplasia. This sample illustrates a positive (+) OL showing a weak staining (score 1 for intensity) in 50% to 75% of cells (score 3 for extension) (final score = 4). **C,** OL from tongue, with hyperkeratosis and no epithelial dysplasia. This sample illustrates strongly positive (++) expression of EGF. It shows very strong labeling (score 4) in more than 75% of the cells (score 4) (final score = 8). **D,** OL of the tongue showing no epithelial dysplasia. Moderate nuclear staining can be detected. A high resolution version of the image is available as eSlide:VM00400.

It has previously been demonstrated that patients with OSCC have lower salivary EGF levels compared with healthy individuals and that the amount of this protein is influenced by certain risk factors, such as tobacco use.[13] Therefore, we hypothesized that salivary EGF levels could be reduced in OL; however, in our study, patients with OL had similar EGF levels compared with controls. We should consider that OL is a highly heterogeneous disease, with diverse clinical presentations and histopathologic features that impact the behavior of OL.[22,23] In this preliminary study, we did not consider these factors in the selection of the OL samples, although most lesions were homogeneous and without atypia. A more homogeneous sample is recommended for future studies.

Both groups in our study were represented by individuals approximately 60 years of age. The potential effect of age in the salivary flux was considered in this study. The total protein content was used to normalize the EGF values for each sample, and there was no difference in the salivary protein content between the patients (4.152 ± 1.118 mg protein/mL saliva) and the controls (4.104 ± 2.213 mg protein/mL saliva) ($P = .317$).

Despite this limitation, the results of the present study suggest that diminished salivary EGF levels are observed only when OSCC is established,[14] not in the premalignant stage. One potential explanation for this effect is negative feedback: In the early stages of carcinogenesis, the "normal" EGF levels may contribute to the molecular and morphologic alterations observed in OL. Thus, as a regulatory response, EGF secretion diminishes upon development of OSCC.

However, the effects of EGF in established cancer should also be associated with the process of ulcer or wound healing. The downregulation of EGF and its receptor, as well as the mislocalization of EGFR in the cytoplasm of keratinocytes instead of at the membrane,

Table IV. Association of clinicopathologic features and immunoexpression of EGF

	Negative, N (%)	Positive, N (%)	P
Gender			
Female	8 (66.7)	4 (33.3)	1.000[*]
Male	10 (71.4)	4 (28.6)	
Age (years)			
≤ 60	10 (90.9)	1 (9.1)	.084[*]
> 60	8 (53.3)	7 (46.7)	
Site			
High risk	2 (33.3)	4 (66.7)	.051[†]
Low risk	16 (80.0)	4 (20.0)	
Aspects			
Homogeneous	14 (66.7)	7 (33.3)	1.000[*]
Heterogenous	4 (80.0)	1 (20.0)	
Number of lesions			
Multiple	16 (88.9)	2 (11.1)	.003[†]
Single	2 (25.0)	6 (75.0)	
Smoking habit			
No	10 (66.7)	5 (33.3)	1.000[*]
Yes	8 (72.7)	3 (27.3)	
Alcohol consumption			
No	8 (66.7)	4 (33.3)	1.000[*]
Yes	10 (71.4)	4 (28.6)	
Epithelial dysplasia[‡]			
Present	4 (66.7)	2 (33.3)	1.000[*]
Absent	14 (70.0)	6 (30.0)	

[*]Chi-square test.
[†]Fisher's exact test.
[‡]Present, high-risk; absent, low-risk.

has been demonstrated in chronic wounds (e.g., cancer ulcers). Moreover, exogenous EGF is readily degraded in the chronic wound environment.[24] These findings may explain the low levels of EGF that are observed in oral cancer but not in OL.

Another point to consider is that EGF effects are triggered in the cell only if it is properly bound to EGFR. It is known that the expression of a high number of receptors or truncated receptors on tumor cells can increase the sensitivity to low concentrations of growth factors.[25] Accordingly, genetic alterations have been identified in *EGFR* genes in human cancers.[26-28] Thus, if OL cells have abnormal EGFR expression or function, they could overrespond to normal EGF levels. In accordance with this hypothesis, Ribeiro et al.[29] demonstrated that EGFR immunoreactivity is common in OL, especially in high-risk lesions. Mahendra et al.[10] also described high EGFR expression in dysplastic lesions.

In the present study, we could not identify a relationship between salivary EGF and EGFR or EGF tissue expression. Surprisingly, Bernardes et al.[30] demonstrated that EGFR expression was not dependent on the gene copy number in OSCC. Metha et al.[28] and Tushar and Ramanathan[31] did not identify mutations in the *EGFR* gene in OSCC. This issue should be clarified in future

genetic studies of EGFR in OL. However, the tissue overexpression of EGF could be insufficient for the autostimulation of cancer progression until ectodomain shedding occurs, which is a process dependent on A disintegrin and metalloprotease (ADAM) proteins.[20] Thus, the EGF expressed in epithelial cells is not necessarily delivered to the saliva.

In the carcinogenic process, epithelial and mesenchymal tissues continuously interact via secreted molecules. In this milieu, inflammation may play a crucial role in the stimulation of dysplastic alterations or the protection of the host against them.[32-34] In line with this role, EGF can synergistically enhance the production of interleukin-8 in human gingival fibroblasts,[35] increase matrix metalloproteinase 1 (MMP-1) and MMP-3 production in these cells,[36] and regulate the expression of genes involved in inflammation and cancer.[37] Interestingly, Chang et al.[38] suggested that inflammation activates the binding capacity of EGF in the gingiva, and the upregulation of EGFR in inflamed gingiva may be associated with a lower EGF concentration in the gingival crevicular fluid. Thus, it appears important to explore the relationship between inflammation and EGF in OL and OSCC.

No significant association was identified between the salivary levels of EGF and the clinical features of OL. Importantly, some features (female gender, location in the floor of the mouth and the tongue, size >200 mm, heterogeneous type, and presence of epithelial dysplasia) have been associated with lesions with a worse prognosis.[39] Thus, we can infer that salivary EGF cannot be regarded as an indicator of high-risk OL at this point. *Leukoplakia* is a clinical term, which is not associated with a specific histopathologic diagnosis. Nevertheless, there was a tendency for patients with OL lesions with dysplasia to present with high salivary EGF ($P = .083$), which is regarded as the most important feature related to OL prognosis. This hypothesis should be confirmed in further follow-up studies with larger sample sizes or in animal models of oral carcinogenesis. These studies would clarify whether EGF contributes to the evolution of OL, thereby favoring cancer development.

An association was identified between the number of lesions and tissue EGF levels, but at this point, this association cannot be explained. An evaluation of all lesions is necessary to confirm this result, and the biopsy of multiple areas within one lesion tipically yields small specimens.

Nuclear EGF immunolabeling was observed and was considered positive in 34.6% of cases and strongly positive in 7.7%. Accumulating evidence suggests that the nucleus is a second site of cellular EGF action. This evidence has been demonstrated by the presence of EGF and EGFR in various types of tissues, including
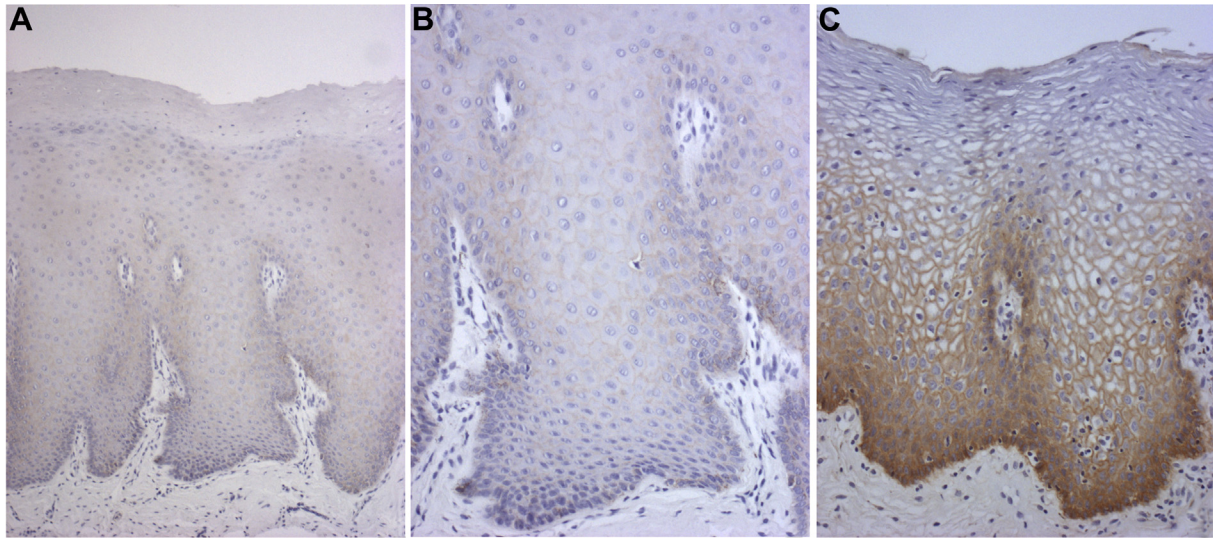
Fig. 3. Epidermal growth factor receptor (EGFR) expression in oral leukoplakia (OL). **A,** OL from retromolar region showing hyperkeratosis and mild dysplasia. This specimen shows a weak homogeneous labeling in >10% of the cells (score 1), thus set as negative. **B,** Closer caption of **A**. Note the weak and homogeneous membranous staining in keratinocytes mainly in the spinous layer. **C,** OL from jugal mucosa showing hyperkeratosis and no dysplasia. This sample shows an intense homogenous labeling in >10% of the cells (score 3), thus classified as positive. A high resolution version of the image is available as eSlide: VM00399.

keratinocytes.[40] Marti et al.[41] demonstrated that EGF and its receptor were found not only in association with the cell membrane but also in the cytoplasm and nuclei of all thyroid tissues investigated. This same pattern was identified in OL, indicating the need for further investigation to elucidate the relationship between the nuclear staining of EGF and the pathogenesis of OL. A membranous distribution of EGFR was observed in 61.5% of cases; this is consistent with the normal location of this receptor.

CONCLUSIONS
Salivary EGF levels in patients with OL do not differ from those in healthy individuals despite the importance of EGF and EGFR in neoplasia. Salivary levels of EGF were not associated with tissue EGF either. So, at this moment, it is not possible to recommend the use of salivary EGF as a biomarker of leukoplakia. Further studies are needed to elucidate the role of EGF in oral carcinogenesis and to assess possible changes in EGF values following the surgical excision of OL.

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Reprint requests:

Maria Cássia Ferreira de Aguiar
Universidade Federal de Minas Gerais
Faculdade de Odontologia, Departamento de Clínica
Patologia e Cirurgia Odontológicas; and Patrícia Carlos Caldeira
Av. Antônio Carlos, 6627
Pampulha, Belo Horizonte, MG, Brazil, CEP: 31.270-901
Cassiafa@ufmg.br; pccaldeira@ufmg.br