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# Repair genes expression profile of MLH1, MSH2 and ATM in the normal oral mucosa of chronic smokers

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ABSTRACT

*Objective:* The aim of this study was to evaluate the effect of chronic smoking on the expression profile of the repair genes MLH1, MSH2 and ATM in the normal oral mucosa of chronic smokers and never smokers.
*Materials and methods:* The sample consisted of thirty exfoliative cytology smears per group obtained from Smokers and Never Smokers. Total RNA was extracted and expression of the MLH1, MSH2 and ATM genes were evaluated by quantitative real-time and immunocytochemistry. The gene and protein expression data were correlated to the clinical data. Gene expression was analyzed statistically using the Student *t*-test and Pearson's correlation coefficient, with $p < 0.05$.
*Results:* MLH1, MSH2 and ATM genes were downregulated in the smoking group compared to the control with significant values for MLH1 ($p = 0.006$), MSH2 ($p = 0.0001$) and ATM ($p = 0.0001$). Immunocytochemical staining for anti-MLH1, anti-MSH2 and anti-ATM was negative in Never Smokers; in Smokers it was rarely positive. No significant correlation was observed among the expression of MLH1, MSH2, ATM and age, number of cigarettes consumed per day, time of smoking during life, smoking history or levels of CO in expired air.
*Conclusion:* The expression of genes and proteins related to DNA repair mechanism MLH1, MSH2 and ATM in the normal oral mucosa of chronic smokers was reduced.


## 1. Introduction

Oral carcinogenesis is a multifactorial and multistep process that involves numerous genetic and epigenetic processes that are modulated by hereditary predisposition and environmental factors (Califano et al., 1996). In the case of oral squamous cell carcinoma (SCC), the most common malignant tumor that affects the structures of the oral cavity (Jerjes et al., 2010), several risk factors have been identified which are mainly related to diet, lifestyle, and habits.

Smoking is the main risk factor for oral SCC, which is an initiator/promoter of this disease (Winn, 2001), since tobacco contains carcinogens that interfere with different stages of carcinogenesis (Scully, Field, & Tanzawa, 2000). In this respect, the smoking habit causes changes in the mechanisms that control cell growth, increasing the proliferative activity of cells in smoking patients even in the absence of clinical lesions (Fontes, Correa, Issa, Brandão, & Almeida, 2008).

A conventional biopsy is the gold standard for the pathological diagnosis of oral malignant diseases. However, exfoliative cytology has been proposed as a complementary method since it is noninvasive, painless, practical, and inexpensive (Almeida, Cabral, & Brandao, 1994). Furthermore, this method provides different types of information about epithelial cells (Lima et al., 2015; Pérez-Sayáns, García-García, Reboiras-López, & Gándara-Vila, 2009), since cells of the superficial layers of the epithelium store information about changes that have occurred during the process from cell maturation to cell desquamation (Papanicolaou GN & Traut, 1941). The cytological study provides information about the changes undergone by cells exposed to genotoxic agents,

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cytogenetic damage (Lima et al., 2010) and changes in oral epithelial maturation (Pavanello, Prado, Balducci, Brandão, & Almeida, 2006). Additionally, it is a suitable technique to obtain samples for the study of genetic alterations (Reboiras-López et al., 2012).

Exfoliative cytology may be useful for the detection and monitoring of early changes in smokers (Fontes et al., 2008). However, the study of oral SCC progression may be restricted to markers that are expressed in superficial layers of the epithelium. Nevertheless, considering the importance in monitoring oral health, the method is indicated as a screening tool for subjects exposed to known carcinogenic agents.

Alterations in DNA repair genes directly influence carcinogenesis since genomic stability depends on the efficiency of DNA repair (Liu, Yin, & Pu, 2007). Several studies suggested that reduced expression of certain DNA repair genes were associated with the risk of environment-related cancers such as lung, head and neck, and colon (Cheng, Spitz, Hong, & Wei, 2000; Cheng, Sturgis, Eicher, Spitz, & Wei, 2002; Morimoto, Tsukada, Kominato, & Tanaka, 2005; Soliman et al., 1998; Wei et al., 1998).

The MutL homolog 1 (MLH1) and MutS homolog 2 (MSH2) genes are involved in the repair of errors in DNA base pairing (Modrich, 1994). The loss of their respective proteins due to hypermethylation in the genes has been described in oral SCC and is reported to be one of the early events of tumor progression (González-Ramírez et al., 2011; Sengupta, Chakrabarti, Roy, Panda, & Roychoudhury, 2007), which varies according to the stimulus (Fernandes et al., 2007). The ataxia telangiectasia-mutated (ATM) gene is a tumor suppressor gene of the group of DNA repair genes (Shiloh & Kastan, 2011). Hypermethylation of the ATM gene has been observed in a significant number of cases of head and neck SCC, in which cases exhibiting reduced or absent expression were more aggressive (Ai et al., 2004).

Experience acquired in previous studies with chronic smoking as a model of oral carcinogenesis process (Lima et al., 2010, 2011; Pavanello et al., 2006), allied to the advantages of cytological samples (Pérez-Sayáns et al., 2010), including gene-expression studies (Reboiras-López et al., 2012) and immunocytochemistry studies (Lima et al., 2015) showed the opportunity to study important genetic changes in carcinogenesis in cytological material. Moreover, few studies assessing carcinogenesis in such early stages, in mucosa with no alteration, are found in the literature. The objective of the present study was to evaluate the effect of chronic smoking on the gene and protein expression profile of MLH1, MSH2 and ATM in the oral mucosa of chronic smokers with no clinical changes in the mucosa, as well as never smokers.

## 2. Materials and methods

### 2.1. Study subjects

Material was collected from two groups, with 30 individuals each: a group consisting of chronic smokers consuming ≥20 cigarettes/day for more than 10 years (Smokers group), who participated in the Outpatient Program for Smoking Treatment of the Heart Institute, University Hospital, University of São Paulo Medical School (INCOR-HCFMUSP). The control group consisted of never smokers (Never Smokes group), seen at the outpatient clinic of the Bioscience and Oral Diagnosis Department of the Institute of Science and Technology, UNESP. The study was approved by the Ethics Committee (Protocol CAAE 07386212.0.1001.0077).

The criteria for inclusion in the groups have been established previously by Lima et al. (2010). The smoking history was calculated in pack-years like previously established by Faria, Botelho, da Silva and Ferreira (2012). Additionally, the concentration of carbon monoxide (CO) in expired air was measured as parts per million (ppm) with a piCO+ Smokerlyzer (Bedfont Scientific, Kent, England) (Issa, Forti, Giannini, & Diament, 1998).

Cytological material was collected from the right buccal mucosa with a Rovers Orcellex Brush® (Rovers Medical Devices, Oss, Netherlands) without the previous use of mouth wash (Fontes et al., 2008). These samples were transported in sterile tubes containing 2 mL Roswell Park Memorial Institute (RPMI) medium (Microvet, Madrid, Spain) at −20 °C, protected from light, and stored at −80 °C. Additionally, smears were collected from each patient with a Cytobrush® and mounted on silanized glass slides for immunocytochemistry.

### 2.2. Quantitative RT-PCR (qPCR)

Total RNA was extracted using a Trizol kit (Ambion, Inc., Carlsbad, CA, USA) as recommended by the manufacturer. The concentration, purity and quality of the RNA was verified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA), through agarose gel electrophoresis (Invitrogen™, Carlsbad, CA, USA) stained by ethidium bromide (Invitrogen™, Carlsbad, CA, USA) and visualization on a transilluminator. The extracted total RNA (1 μg) was treated with DNase I (Turbo DNase Treatment and Removal Reagents – Ambion Inc., Carlsbad, CA, USA) and transcribed into complementary DNA (cDNA) using the SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR Kit (Invitrogen™, Carlsbad, CA, USA), according to the protocols recommended by the manufacturer.

The primers for all genes analyzed in the present study were described and used as indicated by Rentoft, Hultin, Coates, Laurell, and Nylander (2010).

The transcribed cDNAs were amplified for relative quantification of the expression of the MLH1, MSH2 and ATM genes in relation to the concentration of the reference gene (TUBA6). In this research, three reference genes ACTB, GAPDH and TUBA6 were tested in all experimental groups. The obtained results were analyzed at http://www.leonxie.com/referencegene.phpe, and the selected reference gene was TUBA6 (Supplementary data).

The qPCR method was applied to evaluate the amount of the cDNA product in the exponential phase of the amplification reaction. SYBR® Green fluorophore (Platinum® SYBR® Green qPCR SuperMix-UDG Applied Biosystems, Framingham, MA, USA) was used at StepOnePlus™ System (Applied Biosystems, Framingham, MA, USA) device, and the following cycling parameters were used: 50 °C for 2 min, followed by an initial denaturation at 95 °C for 2 min, 40 cycles of 95 °C for 15 s and 60 °C for 30 s. The $2^{-\Delta\Delta CT}$ method was used to analyze the relative changes in gene expression from the quantitative RT-PCR experiment (Livak & Schmittgen, 2001).

### 2.3. Immunocytochemistry

For immunocytochemistry, the smears were collected and immediately fixed by alcoholic spray. Then they were stored at −80 °C (Skoog & Tani, 2011). After, smears were submitted to the streptavidin-biotin method using monoclonal antibodies against MLH1 (diluted 1:50; clone ES05; DakoCorporation, Glostrup, Denmark), MSH2 (diluted 1:50; clone FE11; Dako Corporation, Glostrup, Denmark), and ATM (diluted 1:100; clone 2C1, Pierce, Rockford, USA). The primary antibodies were applied for 1 h at room temperature for anti-MLH1 and anti-ATM and overnight at 4 °C for anti-MSH2 in a moist chamber. Exfoliative cytology smears of leukoplakia lesions with histopathological diagnosis of moderate dysplasia were used as positive control; negative control consisted of slides without the primary antibody (Fernandes et al.,

**Table 1**
Data regarding tobacco consumption. Clinical data (age, number of cigarettes consumed per day, time of smoking during life, levels of CO in expired air and smoking history in the group of smokers.

| | Average | Standard Deviation |
|---|---|---|
| Cigarettes/day | 26.59 | 8.26 |
| Years of consumption | 38.67 | 14.1 |
| CO (ppm) | 11.13 | 6.06 |
| Smoking history (pack-years) | 50.95 | 23.49 |

2007).The immunocytochemical slides were examined under a light microscope at 400× magnification. The criterion was the presence or absence of brown staining in the nucleus of the collected cells. The smears were evaluated qualitatively in all its extension and classified into five categories: (i) negative; (ii) rarely positive; (iii) weakly positive; (iv) moderately positive; (v) strongly positive.

### 2.4. Statistical analysis

The data were analyzed statistically using the GraphPad Prism software (GraphPad Software, Inc., California, CA, USA). The groups were compared using the Student *t*-test, with $p < 0.05$ indicating a significant difference. Associations between gene expression levels and clinical data were explored using Pearson's correlation coefficient.

## 3. Results

Thirty patients per group were studied. Smokers group consisted of 20 men and 10 women with a mean (±standard deviation) age of 56.94 ± 11.1 years (range: 37–80 years). The control group consisted of patients matched for gender and age to Smokers group (54.33 ± 13.08 years, range: 37–81 years). Data regarding tobacco consumption are shown in Table 1.

The gene expression of MLH1, MSH2 and ATM in exfoliated oral mucosa cells was analyzed by qPCR as shown in Fig. 1. MLH1, MSH2 and ATM genes were downregulated in the smoking group compared to the control with significant values for MLH1 ($p = 0.006$), MSH2 ($p = 0.0001$) and ATM ($p = 0.0001$).

Immunocytochemical staining for anti-MLH1, anti-MSH2 and anti-ATM was negative in Never Smokers and Smokers group was rarely positive as illustrated in Fig. 2.

No significant correlation was observed between the expression of MLH1, MSH2 and ATM and age, number of cigarettes consumed per day, time of smoking during life, smoking history or levels of CO in expired air. Pearson's correlation coefficients and *p*-values are shown in Table 2.

## 4. Discussion

In the present study we evaluated the expression profile of MLH1, MSH2 and ATM genes in normal cells of the oral mucosa in Smokers compared to Never Smokers and investigate the presence of proteins related to these genes by immunocytochemistry. These genes are essential for a variety of DNA repair pathways that display aberrant activities. They may influence DNA repair capacity with consequent carcinogenesis development. Hence, a reduction in the expression of these genes may be related to the development of different malignant cancers (Morimoto et al., 2005).

Few studies evaluating the carcinogenesis early stages in normal oral mucosa are available in the literature. This research contributes showing the decrease in MLH1, MSH2 and ATM expression observed in exfoliative cytology obtained from chronic smokers. There are many carcinogens in tobacco (Bartsch et al., 2000) responsible to several tissue changes in the carcinogenesis process (Fontes et al., 2008; Pavanello et al., 2006) as the decrease of repair genes studied.

Smoking is a leading risk factor for cancer development in different organs, including oral SCC (Baric, Alman, Feldman, & Chauncey, 1982), since different carcinogens are present in cigarettes (Bartsch et al., 2000). Therefore, smokers should be monitored carefully considering the series of changes that tobacco can cause in the oral mucosa (Winn, 2001). This biomonitoring, whose objective is the early detection of alterations in smokers, can be performed by exfoliative cytology (Fontes et al., 2008), since the preparation of cytological smears is an easy, rapid and low-cost method (Almeida et al., 1994). Cytology can provide data regarding exfoliated epithelial cells (Pérez-Sayáns et al., 2009), since cells of the superficial layers of the epithelium store information about changes that have occurred during the process of epithelial maturation (Fontes et al., 2008), although these cells are in an advanced stage of maturation and their metabolic activity is low. Cytology is of low risk and discomfort to patients, facilitating detection programs in large populations, permitting an early diagnosis, and reducing costs for healthcare systems (Skoog & Tani, 2011). Cytological samples can be studied in different manners to obtain a better understanding of tissue alterations and can also be used to collect samples for gene expression analysis (Reboiras-López et al., 2012). In the present study, exfoliative cytology was used as a collection method to investigate the expression of DNA repair genes in smokers.

Although DNA synthesis is a highly precise process, errors can occur and the function of DNA repair systems is to identify these errors and to eliminate them, with these systems representing an important protective mechanism in damaged tissue (Modrich, 1994). There are several DNA repair strategies. Mismatch repair is

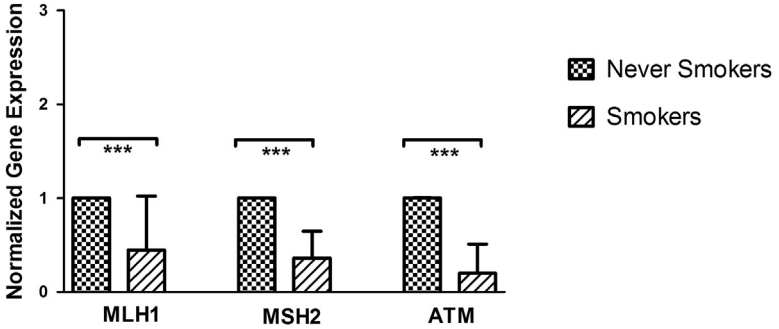


**Fig. 1.** Relative quantification (Log) of the expression of repair DNA genes (MLH1, MSH2 and ATM) in normal cells of the oral mucosa in Smokers and Never smokers using quantitative real-time PCR (qPCR). Values are expressed as the means and standard deviation. Student's *t*-test was used to compare gene expression ($p \leq 0.05$). ***Indicates $p \leq 0.0001$. Each gene was normalized and compared with the control (Never smoker).

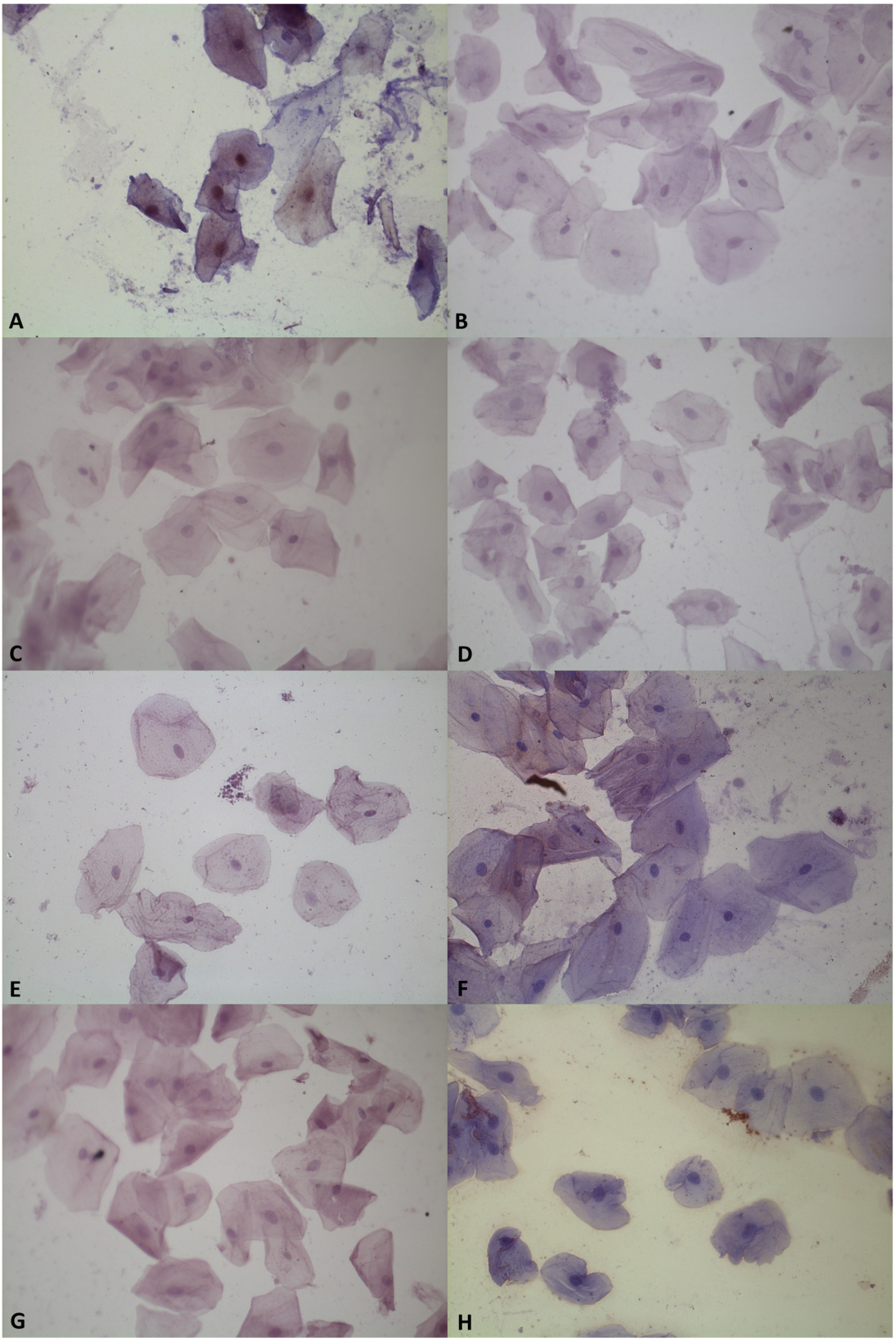


**Fig. 2.** Immunocytochemical staining for anti-MLH1, anti-MSH2 and anti-ATM.A: Positive immunocytochemical control; B: Negative immunocytochemical control. C: Immunocytochemical staining for anti-MLH1 in Never Smokers group; D; Immunocytochemical staining for anti-MSH2 in Never Smokers group; E: Immunocytochemical staining for anti-ATM in Never Smoker group; F: Immunocytochemical staining for anti-MLH1 in Smokers group; G; Immunocytochemical staining for anti-MSH2 in Smokers group; H: Immunocytochemical staining for anti-ATM in Smokers group (400×).

**Table 2**
Results of Pearson's correlation coefficient test among clinical data and the expression levels of the genes studied.

| | | Age | Cigarettes per day | Years of consumption | Smoking history (pack-years) | CO in exhaled air |
|---|---|---|---|---|---|---|
| MLH1 | Pearson's correlation coefficients | 0.3234 | −0.0329 | 0.5098 | 0.3671 | −0.1601 |
| | p-value | 0.2218 | 0.9036 | 0.0537 | 0.1619 | 0.5536 |
| MHS2 | Pearson's correlation coefficients | −0.4000 | 0.2111 | −0.2777 | 0.0089 | 0.4850 |
| | p-value | 0.1116 | 0.5161 | 0.2805 | 0.9730 | 0.0585 |
| ATM | Pearson's correlation coefficients | 0.1627 | 0.1040 | 0.3388 | 0.3203 | 0.1564 |
| | p-value | 0.5471 | 0.7016 | 0.1993 | 0.2265 | 0.5629 |

an intracellular multiprotein process for the repair of non-native DNA structures (Harfe & Jinks-Robertson, 2000; Kolodner & Marsischky, 1999). In the present study, the expression of MLH1, MSH2 and ATM genes were downregulated in the group of Smokers in comparison to Never Smokers. These findings corroborate the ones by Liu et al. (2007); wherein the authors demonstrated that the expression levels of five genes (MSH2, MLH1, XRCC1, XPD, MGMT) were significantly reduced in peripheral blood mononuclear cells obtained from esophageal SCC patients. They suggested the possible molecular mechanism of specific genetic events in tumor progression. Transcriptional silencing of MLH1 and MSH2 and consequent reduced DNA mismatch repair capacity were closely associated with microsatellite instability in cancer (Geisler et al., 2003; Stefansson et al., 2002). The silencing of the two genes appears to be controlled by aberrant hypermethylation of a specific region in the promoter (Grady, Rajput, Lutterbaugh, & Markowitz, 2001; Tzao et al., 2005; Wang et al., 2003).

In order to elucidate the results in gene expression, immunocytochemistry was made to determine the level of protein expression related to the genes analyzed. The results obtained for the three antibodies were rarely observed for both groups analyzed. The weak staining is in agreement with the results described by Theocharis et al. (2011). These authors demonstrated the absence of MLH1 and MSH2 proteins in normal mucosa adjacent to oral SCC lesions. Otherwise, Czerninski et al. (2009) demonstrated positive immunohistochemical staining for proteins MLH1 and MSH2 only in the basal and parabasal layers. With respect to protein ATM, He, Chen and Li (2008) observed in apparently normal oral tissues moderate positive nuclear staining exclusively in the basal layer (He et al., 2008). It should be noted that qPCR is highly sensitive in detecting small amounts of early copies of genetic material. This technique, which is combined with reverse transcription, is therefore effective in quantifying the expression levels of a gene. On the other hand, immunocytochemistry is an important laboratory tool (Skoog & Tani, 2011), which is effective, easily applied and valuable for the identification and classification of tumor cells, showing good agreement with immunohistochemistry (Flens et al., 1990).

No significant correlations were observed between expression of the genes studied and clinical data such as age, number of cigarettes consumed per day, time of smoking during life, smoking history, or CO levels in expired air. This finding may be due to the fact that all smokers studied exhibited high CO levels in expired air, high nicotine dependence, and an elevated smoking history.

In the present study, we observed a reduction in the expression of genes related to DNA repair in exfoliative cells of normal oral mucosa of smokers. Therefore, functional analysis of genetic and epigenetic events involved in the gene expression of the ones studied is required.

## 5. Conclusion

The expression of genes and proteins related to DNA repair mechanism MLH1, MSH2 and ATM in the normal oral mucosa of chronic smokers was reduced.

## Conflict of interest statement

None declared.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.archoralbio.2016.09.006.

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