# Short Communication

# Salivary Levels of TNF-α and IL-6 in Patients with Oral Premalignant and Malignant Lesions

(proinflammatory cytokines / premalignant / malignant lesions)

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**Abstract. The aim of this study was to determine the salivary concentrations of tumour necrosis factor α (TNF-α) and interleukin 6 (IL-6) in patients with premalignant and malignant lesions. The study involved 57 patients who were examined between 2008 and 2010 at the Department of Oral Medicine and Department of Oral and Maxillofacial Surgery of the Medical Faculty, University of Rijeka, Croatia. They were divided into three groups: 19 patients with oral premalignant lesions, 19 with oral squamous cell carcinoma and 19 healthy control volunteers. Whole saliva was collected and investigated for the presence of TNF-α and IL-6 by enzyme immunoassay at the Department of Dentistry and Maxillofacial Surgery, Medical Faculty, University of Graz, Austria. All groups had statistically significant differences in values of TNF-α and IL-6 (P < 0.001). The results suggest that these proinflammatory cytokines are elevated in the saliva of patients with oral squamous cell carcinoma and oral premalignant lesions as compared to controls, which may have diagnostic and/or prognostic significance.**

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Abbreviations: ELISA – enzyme-linked immunosorbent assay, GM-CSF – granulocyte-macrophage colony-stimulating factor receptor, IL-6 – interleukin 6, OLP – oral lichen planus, OPML – oral premalignant lesions, OSCC – oral squamous cell carcinoma, SCC – squamous cell carcinoma, TNF-α – tumour necrosis factor α, VEGF – vascular endothelial growth factor.

## Introduction

Oral lichen planus (OLP) is a complex immunologically mediated mucocutaneous disease. The lesions may become erosive or atrophic or may present in other clinical patterns. Histologically, OLP is characterized by dense band-like lymphocytic infiltrate in the immediate subepithelial region with basal cell destruction. Unlike the clinical behaviour of cutaneous lichen planus, which has essentially no risk of malignant transformation, OLP has a documented risk for malignant transformation (Giesecke et al., 2003). The risk of malignant transformation of OLP was reported from 1.2 % to 3.2 % in follow-up of up to 10 years, but this term still represents a very controversial issue. Although OLP is considered a premalignant lesion by the World Health Organization, it is also argued that OLP is not precancerous and that only OLP-like lesions demonstrating dysplasia present a potential risk of developing into cancer (Epstein et al., 2003).

Squamous cell carcinoma represents more than 90 % of all head and neck cancers. It is an invasive epithelial neoplasm with varying degrees of squamous differentiation that arises from the following anatomic sites: the oral cavity, particularly oral soft tissues including the gingival and alveolar mucosa, floor of the mouth, tongue, soft and hard palates, tonsils and oropharynx (Re et al., 2010). All oral cancers show a strong association with alcohol consumption and tobacco smoking, particularly of cigarettes – in fact, tobacco is thought to be implicated in well over 80 % of cases of oral cancers. Chronic exposure of the epithelial surfaces of the head and neck to these irritants may result in hyperplasia, dysplasia and carcinoma, that is, the development of premalignant lesions that may then undergo malignant change to become an oral cancer (Adeyemi et al., 2011). Although oral lesions are common, predicting which lesions will progress to invasive carcinoma and which will remain stable and have an indolent clinical course is

challenging. Most premalignant lesions clinically present as leukoplakia or erythroplakia, but histologically they may have a wide range of phenotypes such as hyperkeratosis, dysplasia, or carcinoma (Silverman et al., 2001). Early detection efforts, in combination with strategies for prevention and risk reduction, have the potential to dramatically improve clinical outcomes (Marur and Forastiere, 2008).

Tumour necrosis factor (TNF) is a major mediator of inflammation, with actions directed towards both tissue destruction and recovery. While inducing death of diseased cells at the site of inflammation, TNF stimulates fibroblast growth. In malignant disease, high-dose local TNF selectively destroys tumour blood vessels (Koong et al., 2000), but when chronically produced, this cytokine may act as an endogenous tumour promoter, contributing to the tissue remodelling and stromal development necessary for tumour growth and spread. TNF can be detected in malignant and/or stromal cells in human ovarian, breast, prostate, bladder, and colorectal cancer, lymphomas, and leukaemias, often in association with ILs 1 and 6 and macrophage colony-stimulating factor (Burke et al., 1996).

Interleukin-6 (IL-6) is a multifunctional cytokine that is critical for B-cell differentiation and maturation, immunoglobulin secretion, cytotoxic T-cell differentiation, acute-phase protein production, bone marrow progenitor stimulation, and macrophage/monocyte functions (Kishimoto, 2005). Given the reported involvement of IL-6 and its downstream targets in the regulation of cell proliferation, survival, and metabolism, it is not surprising that IL-6 signalling has also been implicated in tumorigenesis (Hodge et al., 2005). Multiple studies have documented high IL-6 levels in the serum of patients with certain carcinomas and have correlated high IL-6 levels with a poor clinical prognosis. These data imply an oncogenic role for IL-6; however, knowledge of the mechanisms governing IL-6 production in tumours and the biological role of this cytokine in tumorigenesis is lacking (Sansone et al., 2007).

The aim of this study was to compare salivary concentrations of TNF-α and IL-6 in patients with oral premalignant lesions, oral squamous cell carcinoma and healthy controls.

## Material and Methods

### *Patients*

Nineteen (19) subjects with OPML (N = 19) and a group with definitively diagnosed OSCC (N = 19) were enrolled into the study along with age-sex matched controls without oral lesions (N = 19). All subjects were informed of the aims and procedures of the study and of the fact that their medical data would be used in research. The Committee for Ethical Approval of Dental Clinic, Medical Faculty, University of Rijeka reviewed the research project and concluded that the proposed project, methodology and research meet the needs of the ethical assumptions and accepted research.

The participants of the study were guaranteed respect to their basic ethical and bioethical principles – personal integrity (independence, righteousness, well-being and safety) as regulated by the Nuremberg Code and the most recent version of Helsinki Declaration. Only those subjects who have given a written permission in the form of informed consent were included. Each subject completed a questionnaire for demographic and health information. The patients were diagnosed clinically and confirmed as having OLP by biopsy, according to the criteria of the WHO.

Patients with chronic inflammatory disease were not considered for the enrolment. Individuals taking drugs that induce hyposalivation, either prescribed or non-prescribed (e.g., anticholinergics, antihistamines, antihypertensives, β-adrenergic blockers), were excluded from the study. None of the subjects were using secretogogues. None of the lesions had been treated in any manner prior to sample collection. Clinical examination was performed according to the standard clinical criteria. Biopsies of the lesions were acquired by standard technique.

The first group consisted of 19 patients (12 women and 7 men) with clinical erosive lichen referred to the Department of Oral Medicine. Diagnosis was confirmed by histopathologic examination. All of the OPML cases who were found to have moderate to severe epithelial dysplasia qualified for enrolment. Eleven patients had moderate epithelial dysplasia and 8 patients were graded as severe epithelial dysplasia. The second group consisted of 19 patients (5 women and 14 men) with histologically confirmed OSCC from the Department of Oral and Maxillofacial Surgery. The control group (from the Department of Oral Medicine) consisted of 19 patients (9 women and 10 men) and none of them had oral lesions.

### *Saliva collection and cytokine assay*

After informed consent had been obtained and medical, dental and social histories taken, the whole unstimulated saliva was collected between 9.00 and 11.00 a.m using standard techniques described by Navazesh (1993). Participants refrained from eating, drinking, using chewing gum, etc., for at least 1 and ½ h prior to evaluation. Saliva specimens were collected from each participant in sitting position. In patients with OPML and OSCC, salivary samples were collected before any therapeutic procedure. Samples were obtained by requesting subjects to swallow first, tilt their head forward, and expectorate all saliva into 50 ml tubes for 5 min without swallowing. The final volume and flow rate of saliva were determined gravimetrically (analytical balance, model WTS-6001, Sartorius Corp., Long Island, NY). Saliva specimens were stored at -80 ºC until the beginning of analysis. For determination of salivary levels of TNF-α and IL-6, the ELISA test (Sigma Immunochemicals, St Louis, MO) was performed according to the manufacturer's instructions, and the re-

sults were expressed in pg/ml. The test was performed in duplicate and repeated three times. Protein content was expressed in pg/ml.

### *Statistical analysis*

Kruskal-Wallis and Mann-Whitney tests were used in statistical analysis in commercial software SPSS 10.0 (SPSS Inc.,Chicago, Ill) with significance level $P < 0.05$.

## Results and Discussion

The demographic characteristics and smoking status of the subjects in study groups are shown in Table 1. The levels of TNF-α and IL-6 were measured in whole saliva samples using ELISA. Differences in salivary concentration of TNF-α between all groups were statistically significant ($P < 0.001$; Table 2). Subjects with OSCC had the highest ($0.739 \pm 0.176$ pg/ml) and control group ($0.013 \pm 0.033$ pg/ml) the lowest concentration of TNF-α ($P < 0.001$ ).

There was also a statistically significant difference between the salivary concentration of IL-6 between all groups, being the highest in OSCC ($0.707 \pm 0.234$ pg/ml) and the lowest in the control group ($0.002 \pm 0.002$ pg/ml) ($P < 0.001$; Table 2). No significant differences in salivary concentrations of TNF-α and IL-6 between smokers and non-smokers were found (Table 3 ).

Prognosis of oral squamous cell carcinoma (SCC) is dependent on early diagnosis. Unfortunately, oral cancer is usually detected when it becomes symptomatic and, at this stage, at least two thirds of the patients present an advanced disease. In many cases, clinicians have difficulty in recognizing patients at high risk of developing oral cancer (Jemal et al., 2008).

Finding early-stage, previously undetected disease may ultimately save lives. Saliva presents a perfect medium to be explored for health and disease monitoring. Moreover, the use of easily accessible biomarkers may prove highly beneficial in large populations or chemoprevention trials.

The present study showed that TNF-α and IL-6 cytokines were detected in the whole saliva of all groups, but the patients with OPML and OSCC have higher salivary concentrations compared with healthy controls. Previous studies have demonstrated that some pro-inflammatory cytokines such as TNF-α , IL-1α, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor receptor (GM-CSF) and vascular endothelial growth factor (VEGF) can be found in significantly elevated quantities in the local milieu of SCC (Rhodus et al., 2005a). There is evidence that these cytokines are produced in dysregulated fashion in oropharyngeal SCC and that they have roles in growth, invasion, interruption of tumour suppression, immune status and even survival.

Higher salivary concentrations of cytokines in oral cancer patients were also reported by other authors. Rhodus et al. (2005b) reported increased concentrations of salivary IL-6 and TNF-α in oral cancer patients and in patients with oral precancerous lesions, while St John et al. (2003) found no significant differences in salivary IL-6 in oral cancer patients compared to healthy controls.

The pathological activities of TNF-α have attracted much attention. For instance, although TNF-α causes necrosis of some types of tumours, it promotes growth of other types of tumour cells. High levels of TNF-α correlate with increased risk of mortality. TNF-α participates in inflammatory disorders of both inflammatory and non-inflammatory origin (Burke et al., 1996). IL-6 is a multifunctional cytokine that was originally characterized as a regulator of immune and inflammatory responses; however, elevated expression of IL-6 has been detected in multiple epithelial tumours (Kishimot., 2005). Many studies have documented high IL-6 levels in the serum of patients with certain carcinomas (i.e., breast, lung, lymphoma) and have correlated high IL-6 levels with a poor clinical prognosis (Hodge et al., 2005). These data imply an oncogenic role for IL-6. It is possible that cytokines with pro-inflammatory and pro-angiogenic activity are produced by squamous cell carcinomas and could contribute to the progression of oral cancer.

*Table 1. Demographic data*

| | OPML | OSCC | Control |
|---|---|---|---|
| **Age** (mean ± SD) | 55.1 ± 5.2 | 54.2 ± 8.4 | 44.1 ± 4.5 |
| **Gender*** | | | |
| Men | 5 (26.3 %) | 12 (63.2 %) | 10 (52.6 %) |
| Women | 14 (73.7 %) | 7 (36.8 %) | 9 (47.4 %) |
| **Smoking**** | | | |
| Active smokers | 12 (63.2 %) | 13 (68.4 %) | 8 (42.1 %) |
| Non-smokers | 7 (36.8 %) | 6 (31.6 %) | 11 (57.9 %) |

*$\chi 2$ test $P = 0.064$.
** $\chi 2$ test $P = 0.221$.

*Table 3. Salivary TNF-α and IL-6 in smokers and non-smokers*

| | Smokers | Non-smokers | P* |
|---|---|---|---|
| TNF-α | 0.511 ± 0.336 | 0.368 ± 0.355 | 0.404 |
| IL-6 | 0.404 ± 0.331 | 0.346 ± 0.366 | 0.122 |

*Mann-Whitney test

*Table 2. Detected values of TNF-α, IL-6 in patients with OLP, OSCC and healthy controls (pg/ml) (mean ± SD)*

| | OPML | OSCC | Control | P* |
|---|---|---|---|---|
| TNF-α | 0.601 ± 0.178 [a] | 0.739 ± 0.176 [b] | 0.013 ± 0.033 [c] | < 0.001 |
| IL-6 | 0.431 ± 0.217 [d] | 0.707 ± 0.234 [e] | 0.002 ± 0.002 [f] | < 0.001 |

*Kruskal-Wallis test. Groups that do not share the same superscripts in rows differ at $P < 0.05$ according to Mann-Whitney test.

In this study salivary concentrations of TNF-α and IL-6 were not affected by smoking. Brailo et al. (2006) also found no differences in the concentrations of salivary IL-6 and TNF-α between smokers and non-smokers with oral leukoplakia and healthy controls. In a study done by Rhodus et al. (2005a) the authors also found no significant difference in these cytokine concentrations between smokers and non-smokers with oral cancer, oral lichen planus and healthy controls. These results indicate that increased salivary concentrations of TNF-α and IL-6 reflect local production of these cytokines in cancer tissue.

The fact that these cytokines in whole saliva were significantly elevated may have diagnostic and prognostic utility as useful biomarkers and indicators of carcinogenic transformation from OPML to cancer.

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