SALIVARY MARKER IL-6 FOR DETECTION OF POTENTIALLY MALIGNANT ORAL DISORDERS: A PILOT STUDY

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ABSTRACT

BACKGROUND: Cytokines and chemokines have been analysed in patients with oral squamous cell carcinoma and potentially malignant disorders as a biomarkers to predict malignant transformation. Cytokines play a major role in modulating the immune response, and are either proinflammatory (IL-1, IL-8, IL-6, TNF-α and TGF-β), or anti-inflammatory (IL-2, IL-4, IL-10, IL-12, and IFN-γ). We selected interleukin-6 (IL-6) because it is a multifunctional interleukin reported to be altered in potentially malignant oral disorders and in malignant lesions. Present study was done to compare concentration of IL-6 in PMD patient with healthy controls.

OBJECTIVES: This study was designed to estimate the levels of salivary IL-6 in potentially malignant disorders (PMDs) (leukoplakia, lichen planus, oral submucous fibrosis (OSF)) and compare them with healthy controls. The aim was to evaluate its efficacy as a potential biomarker for these diseases.

METHODS: The study sample comprised of saliva samples from 12 patients diagnosed with potentially malignant lesions and 12 healthy volunteers. The enzyme-linked immunosorbent assay (ELISA) test was used to measure concentrations of IL-6 levels in four groups: 4 patients with leukoplakia, 4 with lichen planus, 4 with OSF and 12 with healthy controls and the concentration of IL-6 was compared among the PMD patients and healthy individuals.

RESULTS: The levels of salivary IL-6 concentration were found to be significantly elevated in patients with PMDs as compared to the healthy control group ($p<0.0001$). Patients with OSF had significantly higher IL-6 concentrations in their saliva compared to patients with other premalignant lesions.

CONCLUSIONS: Our results suggested that salivary IL-6 can be utilised as a potential biomarker to predict malignant transformation of PMDs. The present study was done with very few sample size. Hence, possible role of IL-6 to predict transition of premalignancy to malignancy needs further research with larger sample sizes with patients having oral malignancy.

CLINICAL RELEVANCE: Saliva as a diagnostic biofluid offers a number of advantages over blood-based testing. The role of IL-6 in PMD if validated further by future research can provide an easy diagnostic tool as well as a prognostic indicator

for patients undergoing treatment. Therefore, it could open up new avenues to find out novel diagnostic tools for the prediction of malignant transformation of PMD.

Key words: Potentially malignant disorders (PMDs), Saliva, Cytokine-Interleukin-6,. ELISA .

LIST OF ABBREVATIONS

ABBREVATION	EXPANSION
PMD	Potentially malignant disorder
IL-6	Interleukin-6
OSF	Oral submucous fibrosis
OLP	Oral lichen planus
OSCC	Oral squamous cell carcinoma
PCR	Polymerase chain reaction
ELISA	Enzyme linked immunosorbent assay
FOM	Floor of the mouth
NOM	Normal oral mucosa
NFκB	Nuclear factor-kappa B
JAK	Janus kinases
STAT	Signal transducers and activators of transcription
MAPK	Mitogen-activated protein kinase

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INTRODUCTION

Oral mucosa represents the initial part of the digestive tract and is exposed to various exogenous toxins. Exposure for longer duration can lead to changes that lead to precancerous lesions or cancers[1]. Precancerous lesions of oral mucosa, known as potentially malignant disorders (PMDs) in recent years consists of a group of diseases namely oral leukoplakia, oral submucous fibrosis (OSF), and oral lichen planus with have a very high malignant transformation rate[2].

The etiology of precancerous lesions of oral mucosa is not well-known[3]. Some risk factors such as tobacco chewing, tobacco smoking, and alcohol play an important role in development of potentially malignant oral conditions. Tobacco chewing is a major risk factor for oral leukoplakia, OSF, and lichen planus. Alchol consumption may increase the risk for oral leukoplakia by 1.5fold and for OSF by2-fold.[2]

Figure 1 Leukoplakia

Oral leukoplakia, the most common PMDs of the oral cavity[4]. Leukoplakia is a term describing "a white lesion of the oral mucosa that cannot be characterized clinically or microscopically as any other defined oral disease entity"[5]. At a World Health Organisation (WHO) workshop held in 2005, it was recommended that oral leukoplakia be defined as "a white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer"[6,7]. OL is classified in two main types: homogeneous type which appears as a flat white lesion and non-homogeneous type which includes speckled, nodular and verrucous leukoplakia[8]. The homogeneous leukoplakia is a uniform, thin white area altering or not with normal mucosa. The speckled type is a white and red lesion, with a predominantly white surface. The nodular type has small polypoid outgrowths, rounded predominantly white excrescences[8]. Verrucous leukoplakia has an elevated, proliferative or corrugated surface appearance. Proliferative verrrucous leukoplakia is a subtype of verrucous

leukoplakia characterized by an aggressive evolution, a multifocal appearance, resistance to treatment, higher degree of recurrence and a high rate of malignant transformation [9]. Leukoplakia has a risk of malignant transformation varying from 0.6% to 18% [10], [11]

Figure 2 Lichen planus

Oral lichen planus, chronic inflammatory disease affecting the oral mucosa with characteristic relapses and remissions[12]. Clinically presents as lesion with radiating whitish gray lines. It is more prevalent in middle-aged females[13]. It appears frequently in all regions of the oral mucosa, mostly noticed in buccal mucosa, gingiva and tongue. They are present bilaterally in most cases [14]. OLP may be seen in six different sub types including Reticular (web like ,fine white striae cross each other in the lesion – sometime referred to as Wickham's Striae), Papular type, Bullous type, Plaque type, Atrophic (areas of erythematous lesion surrounded by reticular components), Erosive or Ulcerative type (area in which erythematous areas are seen surrounded by reticular elements)[14]. The reticular type of oral lichen planus is often asymptomatic [15] only can be seen clinically. Atrophic and ulcerative subtypes have the greater increased malignant transformation risk compared to another subtypes[2].

Oral submucous fibrosis (OSF), a chronic disease of the oral mucosa characterized by inflammation and a progressive fibrosis of the lamina propria and deeper connective tissues[16] . Although it is occasionally preceded by formation of vesicles[17]. OSF is highly prevalent in people in Indian subcontinents and South-East Asia[18]. Its etiology is multifactorial but arecoline in the areca nut is the main causative

Figure 3 Oral submucous fibrosis

agent in initiating the disease process. OSF is clinically characterized by a early sign and a symptoms of burning sensation, vesiculation and ulceration in the oral cavity and lately followed by blanching of the oral mucosa. This results in to increasing stiffeness and marked rigidity of the tissues leading to reduced mouth opening and inability to eat[19],[13],[20].

Most of the oral cancerous lesions are usually preceded by PMDs, hence early detection of premalignant lesions and oral cancer is very important for the prevention of oral cancer. The rate of malignant transformation of such potentially malignant disorders (PMDs) into OSCC fluctuates based on multiple factors like population, gender, habits and the dysplasia grade .Effectively addressing such premalignant disorders at an early stage facilitates to arrest progression into oral squamous cell carcinoma (OSCC). Amongst the established PMDs, early diagnosis of high risk PMDs is of utmost priority to truncate both morbidity and mortality rates[21].

Various modalities such as oral cavity examination, supravital staining, oral cytology and optical technologies including spectroscopy, fluorescence spectroscopy, elastic scattering (reflectance) spectroscopy, Raman spectroscopy, fluorescence imaging, optical coherence tomography, narrow-band imaging, and multimodal optical imaging may be used[2]. Though biopsy is the standard for diagnosis of potentially malignant oral disorders (PMDs), it is not convenient for screening and follow-up due to its invasive nature, high cost, and need for specially trained medical personnel and equipment. Moreover, the current tools of diagnosis are not enough for detecting high risk PMDs (potentially

malignant disorders) or in post-treatment phases during follow-up, as DNA mutations have been observed even in epithelial cells with no evidence of histopathological changes[22]. Traditionally, grade of oral epithelial dysplasia is the most commonly used indicator to determine the risk of malignant transformation of PMDs. However, this histologic method is inadequate and may involve subjectivity resulting in inaccurate results[23]. Moreover, studies have shown a significant number of lesions that lack dysplastic changes microscopically before progression into OSCC[21] . Thus, it is of utmost importance to develop newer, non-invasive and easy to use diagnostic medium and tools for the detection of PMDs. The detection of discriminatory biomarkers in saliva samples is considered to be the most promising answer at this stage[22].

Recently, accumulating evidence has suggested that the nuclear factor-κB (NF-κB) dependent cytokines play a significant role in the pathological behavior of tumors[24],[25]. The levels of interleukin (IL)-6, IL-8 are elevated in saliva , serum and tissue specimens as well as other cytokines induced as part of the acute phase response can act as diagnostic tools for detecting oral cancerous and precancerous lesions and condtions [26] ,[27],[28].

AIM & OBJECTIVES

Aim:

To detect the presence of salivary biomarkers to aid in diagnosis and therapeutic modalities of PMDs.

Objectives:

1. To assess the level of IL-6 in the saliva samples of PMDs patients
2. To compare the level IL-6 in the saliva samples of PMDs patient and healthy individuals

REVIEW OF LITERATURE

Saliva offers some distinctive advantages when used for diagnosis of disease. Whole saliva can be collected non-invasively, and by individuals with limited training, including the patient. No special equipment is needed for collection of the fluid. Further, analysis of saliva may provide a cost-effective approach for the screening of large populations. Advances in the use of saliva as a diagnostic fluid have been affected by current technological developments: enzyme–linked fluorescence technique, Western blot assays, polymerase chain reaction (PCR)[29].

Cytokines play a major role in modulating the immune response, and are either proinflammatory (IL-1, IL-8, IL-6, TNF-α and TGF-β), or anti-inflammatory (IL-2, IL-4, IL-10, IL-12, and IFN-γ). The nucleus of any cell has the ability to secrete cytokines, but its main is macrophages and helper T cells [30]. Among the cytokines, interleukins have a crucial function and are implicated in oral PMDs.

IL-6 is a multifunctional cytokine with growth-promoting and anti-apoptotic activity. There is evidence that IL-6 regulates activation of the Janus kinases (JAK) and signal transducers and activators of transcription (STATs), which then stimulate pathways involving mitogen-activated protein kinase (MAPK), which in turn supports PMDs development [31] . IL-6 is synthesized in acute inflammatory responses that contribute to host defense. It is involved in the processes of controlling the immune response,inflammation, hematopoiesis,and tumorigenesis. Elevated IL-6 levels may lead to disturbances in immune responses. IL-6 is involved in the regulation of tracking of lymphocytes through the lymph node after developmental stimulation[32] . Gabayn found that IL-6 contributes to the induction of the transition from acute to chronic inflammation through secretion of the monocyte chemoattractant protein-1 (MCP-1) [33]. Salivary IL-6 mRNA was quantified by real-time quantitative PCR. Salivary IL-6 protein concentration was measured by enzyme-linked immune-sorbent assay. IL-6 protein expression in tumor samples was investigated by immunohistochemistry [34].

A study conducted by **Rhodus et al.,** in **2005** manifested that cytokines are elevated in tissue culture of oral squamous cell carcinoma (OSCC) cell lines. The purpose of this study was to determine the level of several inflammatory, NF-Kb dependent cytokines in whole unstimulated saliva (WUS), in subjects with OPML (oral potentially malignant lesion) as compared to those with diagnosed OSCC.

Subjects (n = 13) with OPML, OSCC (n = 13), and age–sex matched controls without oral lesions (C) (n = 13) were enrolled. The mean age was 58.7 years. WUS was collected by standard techniques for 5 min. WUS samples were centrifuged and the cytokine analysis was performed on the supernatants by ELISA .The cytokines analyzed were: TNF-alpha, interleukin-1, interleukin-6, and interleukin-8 (TNF-a, IL-1, IL-6, and IL-8). There was a significant increase in the levels of all cytokines in the saliva of the OPML as compared to controls, and a significant difference in the cytokines of OSSC saliva compared to the OPML and controls. These results suggest that these proangiogenic, proinflammatory cytokines are elevated in the saliva of patients with OSSC and OPML as compared to controls, which may have diagnostic and/or prognostic significance[35].

Another study done by **Katakura et al. (2007).,** examined expression of 4 kinds of cytokine in saliva. The authors measured the levels of interleukins (IL-1β, -6, -8) and osteopontin were measured by ELISA using whole saliva samples. Expression of the 4 cytokines was higher in patients with oral cancer than in healthy controls. The difference was significant in IL-6, in particular. Their results suggested that saliva offers a potential target for a screening test aimed at detection of precancerous lesions[36]. Serum IL-6 levels proved to be a significant independent predictor of recurrence as well as poor survival in a longitudinal, prospective cohort study by **Duffy et al.,** in **2008**[37]

Sharma et al (2010)., Suggested that salivary interleukin-6 (IL-6) as a marker of malignant progression was undertaken in patients with leukoplakia having coexisting periodontitis (n=20), periodontitis patients without leukoplakia (n=20), and healthy controls (n=20) by competitive enzyme-linked immunosorbent assay. Results shown that elevation of IL-6 levels in leukoplakia with coexisting periodontitis and in periodontitis patients when compared to healthy control ($P<0.001$).Within the leukoplakia group, IL-6 level was found to be increased with increase in the severity of dysplasia. The use of tobacco was seen to play a significant role in the elevation of salivary IL-6[38].

According to **Prasad and M. McCullough (2013).,**Chemokines have been showned to be important in both inflammation and carcinogenesis and are able to be measured in saliva with relatively robust methods including enzyme-linked

immunosorbent assays (ELISA). Thus it has been hypothesized that patients with oral cancer and oral potentially malignant lesions will have elevated levels of specific chemokines in oral fluids and that this may be used as a marker of both the early detection of malignant disease and progression to malignancy[39].

In **2013, Punyani & Sathawane** reported that Interleukin 8 (IL-8) is a pro-angiogenic, proinflammatory mediator that belongs to the family of chemokines. Due to its pro-angiogenic characteristic, it may play a vital role in tumour angiogenesis and progression. This study was designed to estimate the levels of salivary IL-8 in oral precancer and oral squamous cell carcinoma (OSCC) patients and compare them with healthy controls. The aim of their study to evaluate its efficacy as a potential biomarker for these diseases. Each group comprised 25 individuals. The salivary IL-8 levels were determined by enzyme linked immunosorbent assay. The levels of salivary IL-8 were found to be significantly elevated in patients with OSCC as compared to the precancer group ($p<0.0001$) and healthy controls ($p<0.0001$). However, the difference in salivary IL-8 concentrations among the precancer group and controls was statistically non-significant (p00.738). Their results suggested that salivary IL-8 can be utilised as a potential biomarker for OSCC. Salivary IL-8 was found to be non-conclusive for oral premalignancy[40].

Czerninski1 et al., in **2014** emphasized that improved clinical detection of PMD could enhance the ability to prevent advanced malignancy. Their study suggested that PMD exhibit features that can raise suspicions during a conventional oral examination or microscopic analysis and currently there is no way to evaluate the risk of a PMD patient to transform to malignancy[41]. In another study conducted by **Abdel-Haq., (2014)** to assess the concentrations of interleukin-6 (IL-6) and neopterin in saliva and serum of patients with lichen planus (including reticular and erosive form of oral lichen planus) and to compare them with the concentrations observed in healthy controls. The study material comprised serum and saliva samples from 56 patients diagnosed with lichen planus and 56 healthy volunteers. The ELISA test was used to measure concentrations of IL-6 and neopterin in the serum and saliva of the study participants. The concentrations of IL-6 in saliva and serum of patients with lichen planus were significantly higher than in controls ($P = 0.0002$; $P < 0.0001$). The difference remains significant after adjustment for gingivitis and age. Patients with atrophic-erosive oral lichen planus had significantly higher IL-6 concentrations

in their saliva compared to patients with reticular form of disease (P = 0.01). The concentrations of neopterin were significantly higher in the serum but not in saliva of lichen planus patients vs. controls (P <0.0001). Serum levels of proinflammatory cytokines IL-6 and neopterin are increased in lichen planus as well as the salivary concentrations of IL-6. The differences observed in IL-6 levels in patients with erosive-atrophic forms of oral lichen planus may indicate a substantial role played by the cytokine in the disease[42].

In a study conducted by **Rajkumar et al.,** in **2014** reported that clinical utility of salivary interleukin (IL)-8 in differential diagnosis of potentially malignant lesion (PML) and oral squamous cell carcinoma (OSCC) under high oral cancer prevalent region. Saliva and blood samples were collected from 100 subjects in each group of OSCC, PML and healthy control subjects. Serum and salivary IL-8 levels were measured by ELISA. Saliva and blood samples were collected from 100 subjects in each group of OSCC, PML and healthy control subjects. Serum and salivary IL-8 levels were measured by ELISA.· Their study confirms that salivary IL-8 can be discriminatory between PMDs and OSCC.[43]. A study conducted in 205 patients included 54 oral lichen planus (41 to 65 years), 50 oral leukoplakia (42 to 65 years), 51 oral submucous fibrosis (41 to 65 years), and 50 healthy controls (42 to 65 years) by **Kaur & Jacobs.,** in **2015** reported that salivary and serum cytokines were also elevated when analyzed in oral precancerous lesions. Thus, salivary and serum IL-8, IL-6, and TNF-α levels might act as diagnostic markers for detection of oral precancer[28] .

The sample of 75 cases was divided into three groups of 25 patients each: group I: oral leukoplakia; group II: OSCC; group III: control group. Saliva samples were collected by simple drooling method and the concentration of IL-6 was determined by using ELISA (enzyme-linked immunosorbent assay) technique conducted by **Selvam and Sadaksharam.,** in **2015** found that increase in salivary IL-6 in leukoplakia and OSCC might point out its local production by the tumor cells. The difference in its levels between these two lesions might indicate the progression of precancer to cancer[44].

Kaur and Jacobs., in **2015** reported that to identify salivary and serum concentrations of interleukin (IL)-8, IL-6, and tumor necrosis factor alpha (TNF-α) in

patients with oral lichen planus, oral leukoplakia, oral submucous fibrosis, and healthy controls. Oral lichen planus, oral leukoplakia, and oral submucous fibrosis cases were diagnosed using histopathological analysis. Salivary and serum cytokine concentrations were measured using enzyme-linked immunoassay kits in all subjects. Their result of study suggested that the levels of serum and salivary TNF-α, IL-6, and IL-8 were statistically significantly increased in oral leukoplakia, submucous fibrosis, and lichen planus in contrast to normal healthy subjects ($P<0.05$). Serum and salivary correlation analysis revealed strong and highly significant correlations for TNF-α, IL-6, and IL-8 in all groups ($r=0.72-0.82$, $P<0.05$). Thus, salivary and serum IL-8, IL-6, and TNF-α levels might act as diagnostic markers for detection of oral precancer [28].

Dineshkumar et al., (2016) Proposed the diagnostic utility of serum and salivary interleukin 6 (IL-6) levels in the differential diagnosis of potentially malignant lesions and conditions (PMLs/PMCs) and oral squamous cell carcinoma (OSCC) in a high oral cancer prevalence region. Salivary and blood samples were collected from 100 participants in each group (OSCC, PMLs, and healthy controls). Serum and salivaryIL-6 levels were measured by enzyme-linked immunosorbent assay. Significant differences in IL-6 concentration were noted between OSCC and PML/C patients in both serum and saliva, with salivary levels being 2 to 3 fold higher than serum values in all the groups . Their results of the study suggested that the pro-inflammatory cytokine, IL-6, is elevated in the saliva of patients with OSSC compared to PMD and controls, and thus may prove to have diagnostic and/or prognostic significance[45].

Bagan et al., in **2016** Reported that saliva is very easy to collect and might be a useful complementary test material for determining the diagnosis of PVL. In this regard, they found that both salivary and serum IL-6 were elevated compared to controls ($p<0.05$), with salivary levels better able to distinguish PVL from controls . In addition, when they analysed the grades of the verrucous area in the oral cavity, there was a significant ($p<0.05$) difference, with the highest salivary IL-6 being correlated with the greatest verrucous extent[46].

Qin et al., (2017) observed in the oral cavity, saliva shows great promise as a noninvasive and sensitive marker for many systemic diseases. Biomarkers are being used as diagnostic or monitoring tools for a wide variety of diseases, including

systemic lupus erythematosus, Sjögren disease, Behçet disease, and autoimmune blistering disorders, as well as premalignant and malignant lesions of the mouth[47].

Khurshid et al., in **2018** developed OraSure and Saliva-Sampler saliva collection devices , a number of additional saliva collection tools have been introduced into the commercial market. This includes Salivette (Sarstedt, Germany), Quantisal (USA), modified version of Saliva-Sampler device for drug monitoring, Salimetrics Oral Swab (SOS, California, USA), the Saliva Collection System (SCS, Greiner Bio-One, Austria), and the OraSure Oral Fluid Drug Collection device (OraSure Technologies, Bethlehem PA, USA)[48].

Mozaffari et al., in **2018** suggested that the higher levels of IL-8 in saliva compared with serum suggest that measurement of this marker in saliva may be more useful than serum measurements for determining therapeutic and diagnostic aims[49]. **Mozaffari et al.,** in **2018** also observed that higher levels of IL-6 in saliva compared with serum suggest that measurement of this marker in saliva may be more useful than serum for diagnostic and therapeutic aims[50].

Sarode et al.,(2019) in their review, mentioned that in a markers can be used for risk assessment of malignant transformation in patients with OPMDs as well as for prophylactic conciliation and fair management of the high-risk OPMD patient group[21].

Gug et al., in **2019** stated that saliva represents a very interesting biological fluid with high potential for biochemical, toxicological and immunological diagnostics and health monitoring. Unlike blood sample, which is prone to clotting, saliva is much easier to handle and its sampling is non-invasive, requiring less pre-analysis manipulation. The most representative immunological techniques used in salivary investigation and analysis are enzyme-linked immunosorbent assay (ELISA), which quantify specific antibody and cytokines in gingival crevicular fluid, and saliva; flow cytometry, used especially for the characterization of T cells from peripheral blood and gingival tissues; immunohistological analysis of the inflammatory cell infiltrate in gingival tissues; RIA; and immunoblotting[51] .

In a recent study conducted by **Babiuch et al.,** in **2020** revealed the immunohistochemical analysis significantly higher expression of IL-8 in OSCC specimens and TNF-alpha in OSCCs and OPMDs with dysplasia as compared to

NOM. Moreover, expression of TNF- alpha was significantly higher in oral leukoplakia and oral lichen planus without dysplasia, whereas expression of IL-8 only in oral leukoplakia without dysplasia in comparison with NOM. Salivary concentrations of all evaluated cytokines were significantly higher in patients with OSCC than in controls. Moreover, levels of IL-8 were significantly higher in saliva of patients with OPMDs with dysplasia as compared to controls and in OSCC patients as compared to patients with dysplastic lesions. There was also significant increase in salivary concentrations of IL-6, IL-8 and TNF-alpha in patients with OSCC as compared to patients with OPMDs without dysplasia[52].

Martina et al.(2020).,carried out to provide an overall perspective of salivary biomarkers concerning oral diseases such as lichen planus, oral cancer, blistering diseases, and psoriasis. Saliva has proved to be a promising substrate for the early detection of oral diseases and the evaluation of therapeutic response. However, the wide variation in sampling, processing, and measuring of salivary elements still represents a limit for the application in clinical practice[53].

Roi et al., (2020) analysed that cytokines directly involved in inflammation and immune response, the role of salivary cytokines in tumor growth and progression linked them to the incidence of oral malignant lesions[54]. Recently, A relevant study was done by **Singh et al.,**in **2020** evaluated 3 protein markers, IL-1β, IL-8 and LGALS3BP using ELISA, from unstimulated saliva samples. Among these LGALS3BP was significantly elevated specifically in early stage OSCCs and PMDs[22].

MATERIALS AND METHODS

Null hypothesis

Salivary Markers like IL-6 will not be detected in the saliva of PMDs.

Study setting

This study was approved by Institution Ethical Committee (IEC) of KMCT Dental Collage. According to ethical principles, a written and informed consent was obtained from all the study participants to confirm that they are fit to participate in the study before collecting saliva.

Study design

A case control study was conducted on PMDs patients who enrolled at out patient department of KMCT Dental Collage during January 2021 to May 2021. Study subjects was recruited by professionally qualified, well trained and experienced Oral Pathologists. The demographic details and information on previous history were collected.

Sample size

n = Z2 1-α/2 ×SN× (1-SN)

L2 × (prevalence)

where *n* = required sample size,

SN = anticipated sensitivity,

SP = anticipated specificity,

α = size of the critical region (1 – *α* is the confidence level),

*z*1-α/2 = standard normal deviate corresponding to the specified size of the critical region (α)

Ref article: Validation of Salivary Marker, IL1β, IL-8 and Lgals3bp for Detection of Oral Squamous Cell Carcinoma in an Indian Population Prerana Singh, Jitendra K.V erma & Jayant Kumar Singh

L = absolute precision desired on either side (half-width of the confidence interval) of sensitivity or specificity.

n = 24

Sample groups

A total of 24 cases were included in the study. The case was selected from the Department of Oral Medicine and Radiology, KMCT Dental College and Hospital, Calicut, India. All the patients were in the age group of 21–80years. They were divided into two groups consisting of 12 cases each:

Group I (study group):

Patients with clinically diagnosed and histopathologically proven PMDs like oral leukoplakia, oral lichen planus and oral sub mucous fibrosis

Group II (control group):

Healthy subjects free of any habits and systemic diseases.

Inclusion criteria:

- Patient with oral leukoplakia
- Patient with lichen planus
- Patient with oral submucous fibrosis

Exclusion criteria:

- Distant metastasis
- Secondary malignancies
- Cardiac insufficiency or myocardial infarction within the last 6 months
- Any systemic or topical treatment suppressing the immune system such as steroids or other immunosuppressive drugs
- Pregnant and lactating subjects
- Patients who did not display any alcohol consumption, tobacco use, prosthetic use and the primary site of the carcinoma

Sample collection

The unstimulated saliva was collected from patient between 8.00 am and 10.00 am. The patients were asked to rinse their mouth thoroughly with water 10 min before saliva collection, and they were asked to spit out or swallow saliva already present in the mouth. After the individuals were comfortably seated and after a few minutes of relaxation, they were trained to avoid swallowing

saliva and asked to lean forward and drool all the saliva they produced into a vial using a custom-made saliva collecting funnel over a period of 5–10 min. Sufficient amount of saliva was collected. Once collected, saliva containing vials was stored in a portable ice carrier box and immediately transferred to the laboratory. Stored saliva was melted and transferred to test tubes. They were subjected to centrifugation for 20 minutes at the speed of 2000-3000 r.p.m to remove debris. The supernatants were carefully drawn using micropipettes and transferred to Eppendorf tubes which were stored at -20°C until analysis .

IL-6 Estimation

The concentration of salivary IL-6 quantified by commercially available ELISA kit(Wuhan Fine Biotech, China). The assay was carried out according to the manufacture's instruction. Briefly, the kit was based on sandwich ELISA(enzyme linked immunosorbent assay) method. Components of the kit were allowed to equilibrate for 20 minutes at room temperature before use. Set standard, test samples(diluted at least ½ with Sample Dilution buffer), control (blank) wells on the pre-coated microplate respectively, wash plate 2 times before adding standard, sample and control wells. Aliquot 100ul of standards of zero tube, 1st tube, 2nd tube, 3rd tube, 4th tube, 5th tube, 6th tube and add sample dilution buffer (blank) into the standard wells. Add 100ul of properly diluted sample to each and and seal the plate with cover and incubate for 90 minutes at 37 degree celsius. Aspirate and wash plate 2 times with wash buffer. Add 100ul Biotin –labeled antibody working solution to each well, cover the plate and incubate for at 37degree Celsius for 60 minutes, aspirate and wash plate 3 times with wash buffer and let the wash buffer stay in the wells for 1-2 minutes each time.Add 100ul HRP-Streptavidin conjugate (SABC) working solution into each well and incubate at 37 degree celsius for 30 minutes. Aspirate and wash plates 5 times with wash buffer, and let the wash buffer stay in the wells for 1-2 minutes each time. Add 90ul TMB (chromogen substrate) solution into each well, cover the plate and incubate at 37 degree celsius resulting in the progressive development of a blue coloured complex with the conjugate. The colour development was then stopped by the addition of stop solution (50ul) turning the resultant final

product yellow. The intensity of colour developed is proportional to the IL-6 present whichwas measured in a microplate reader (Rayto ELISA plate reader) at a wavelength of 450nm. The optical density obtained was then used for calculation of IL-6 present in each sample. The detection range of the kit was from a minimum of 4.688- 300pg/ml. The results were expressed as pg/ml of saliva.

Expected outcome

We are expecting an increase in IL-6 level in salivary samples of PMDs patients.

Statistical methods:

All statistical procedures were performed using Statistical Package for Social Sciences (SPSS) 20.0. Calculations for power (80%) of study was performed before the commencement of the study. All quantitative variables expressed in mean and standard deviation. Qualitative variables expressed in percentages. Shapiro-Wilk test was used for testing the normality assumption of the quantitative data. Chi square test for qualitative and One way ANOVA for quantitative variables was used for association between variables. Probability value ($p < 0.05$) was considered statistically significant.

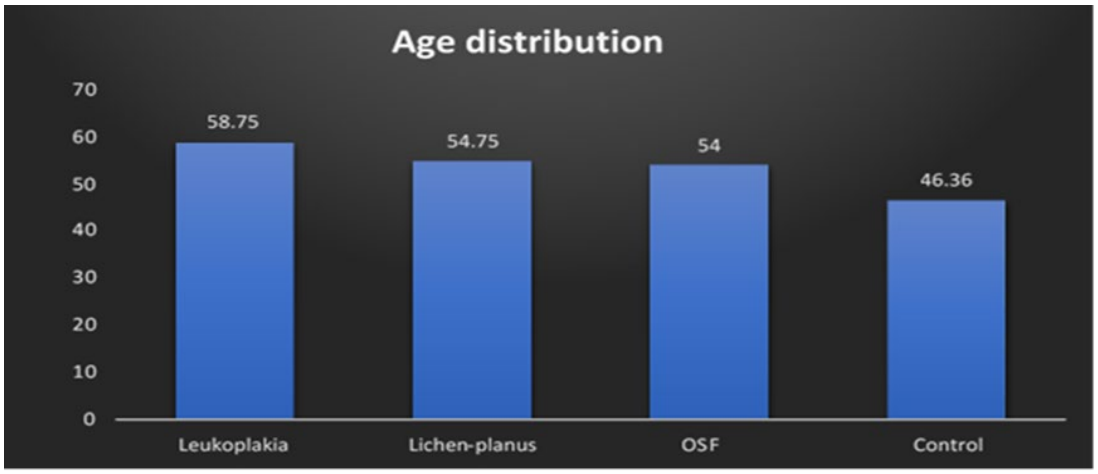
RESULTS

The samples of 24 cases were divided into two groups, 12 premalignant lesions which consisted of 4 each of leukoplakia, lichen planus and OSF out of 24 and 12 healthy subjects are included. The age of patients ranged from 52-65 years (group1 four cases) (mean : 58.75 , SD: 5.56), from 49-60 years (group 2 four cases) (mean : 54.75, SD: 4.78), from 48-63 years (group 3 four cases) (mean: 54.00, SD: 6.37), from 31-58 (group 4 twelve cases) (mean: 46.36, SD: 7.51).(Table 1 and graph 1).

Table 1: Age distribution in all four groups

	Mean	SD
Leukoplakia	58.75	5.56
Lichen-planus	54.75	4.78
OSF	54.00	6.37
Control	46.36	7.51

Graph 1:

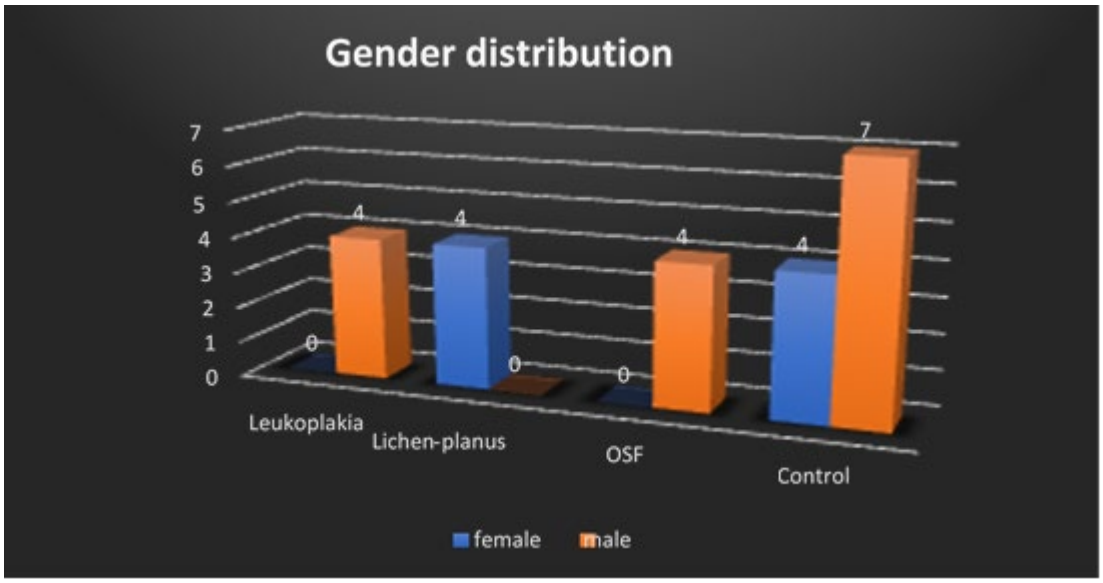


When comparing gender predilection for premalignant lesions, leukoplakia and OSF showed in male predominance and lichen planus showed in female predominance (Table 2 and graph 2).

Table 2: Gender distribution

	Female **n**	**Male** **n**
Leukoplakia	0	4
Lichen-planus	4	0
OSF	0	4
Control	4	8

Graph 2



For leukoplakia 25% cases shows association with smoking, 25% cases shows association with smoking and alcohol, 25% cases shows association with smoking and betel quid, 25% cases shows association with smoking, alcohol and betel quid. For lichen planus, there is no association with habits. For OSF, 25% cases shows association with smoking and alcohol, 50% cases shows smoking and betel quid and 25% cases shows smoking, alcohol and betel quid. The P-value for the association is shown in table 3, it was not statistically significant.

Table 3: Association of habits in different groups

	Smoking n(%)	Smoking and alcohol	Smoking and betel quid	Smoking, Alcohol and betel quid	None	P value
Leukoplakia	1(25)	1(25)	1(25)	1(25)	0	0.09
Lichen-planus	0	0	0	0	4(100)	
OSF	0	1(25)	2(50)	1(25)	0	
Control	0	0	0	0	12(100)	

Of the 24 cases, eight showed the premalignant lesions from buccal mucosa, in which leukoplakia represented 50%, lichen planus 75% and OSF 75%, one cases from floor of the mouth (FOM) for leukoplakia(25%),one cases from gingiva for lichen planus(25%), one cases from mandibular mucosa for leukoplakia(25%) and one cases from tongue (25%). The P-value for association between site of lesion is shown in table 4, it was also not statistically significant.

Table 4 : Site of lesion

	Buccal mucosa n(%)	FOM	Gingiva	Mandibular mucosa	Tongue	None	P value
Leukoplakia	2(50)	1(25)	0	1(25)	0	0	0.09
Lichen-planus	3(75)	0	1(25)	0	0	0	
OSF	3(75)	0	0	0	1(25)	0	
Control	0	0	0	0	0	12(100)	

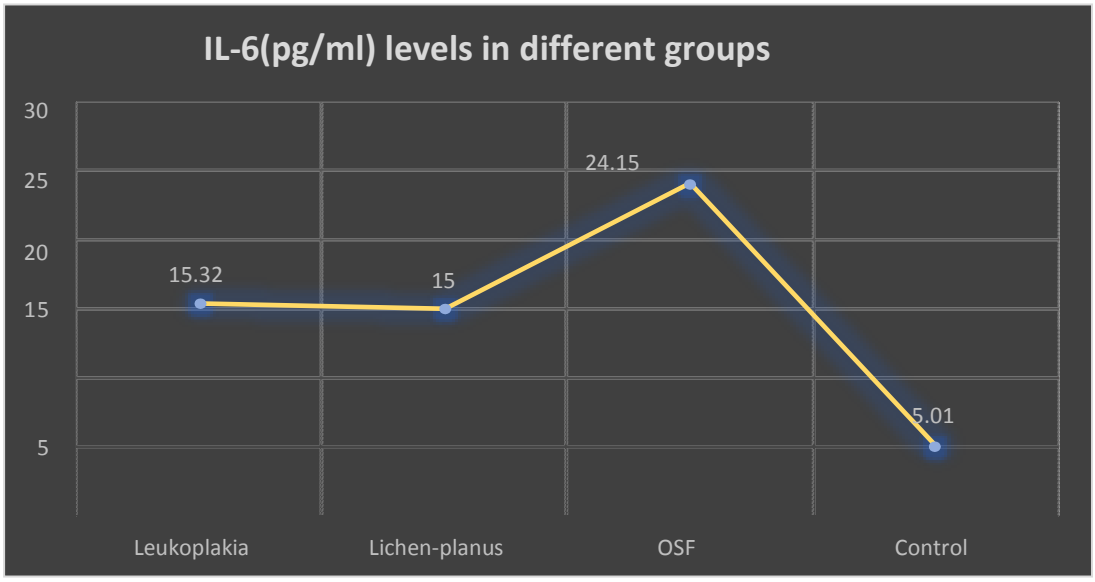
Salivary IL-6 level

The patient with leukoplakia showed 15.32+_3.88 pg/ml (mean and SD) for the concentration of salivary IL-6, for lichen planus 15.00+_2.86 pg/ml, for OSF 24.15+_5.50 pg/ml, and 5.01+_3.06 pg/ml for control subjects respectively. One way ANOVA for IL-6 levels among the four groups was compared, the results were statistically highly significant (P<0.001). The levels of IL-6 significantly higher in in PMDs when compared to control group (Graph. 3). Salivary IL-6 also showed a significantly higher level in OSF compared to other PMDs patient. The exact P-value for the IL-6 levels in different groups shown in Table 5.

	Mean	SD	P value
Leukoplakia	15.32	3.88	0.001**
Lichen-planus	15.00	2.86	
OSF	24.15	5.50	
Control	5.01	3.06	

#p value <0.05 is statistically significant; ** <0.001 is statistically highly significant

Graph 3



Multiple comparisons was done by using LSD, it is found that differences observed between the four study groups showed that each group was significantly different with respect to each other as demonstrated by post hoc test.(Table. 6).

Table 6: Multiple / Post Hoc Group Comparisons in ANOVA

Multiple Comparisons						
LSD						
		Mean Difference (I-J)	Std. Error	P value	95% Confidence Interval	
					Lower Bound	Upper Bound
Leukoplakia	Lichen-planus	.32500	2.58817	.901	-5.0921	5.7421
	OSF	-8.82500*	2.58817	**.003**	-14.2421	-3.4079
	Control	10.32227*	2.13711	**.000**	5.8492	14.7953
Lichen-planus	Leukoplakia	-.32500	2.58817	.901	-5.7421	5.0921
	OSF	-9.15000*	2.58817	**.002**	-14.5671	-3.7329
	Control	9.99727*	2.13711	**.000**	5.5242	14.4703
OSF	Leukoplakia	8.82500*	2.58817	**.003**	3.4079	14.2421
	Lichen-planus	9.15000*	2.58817	**.002**	3.7329	14.5671
	Control	19.14727*	2.13711	.000	14.6742	23.6203
Control	Leukoplakia	-10.32227*	2.13711	.000	-14.7953	-5.8492
	Lichen-planus	-9.99727*	2.13711	.000	-14.4703	-5.5242
	OSF	-19.14727*	2.13711	.000	-23.6203	-14.6742
*. The mean difference is significant at the 0.05 level.						

DISCUSSION

Cytokines are a group of small, mainly secreted proteins that affect the behaviour of cells in a diverse number of ways. The binding of cytokines to specific cell membrane cytokine receptors can induce a number of activities within the cell, such as growth, differentiation, or death[39]. Most cytokines have pleiotropic effects; however, some are generally considered as pro-inflammatory, such as interleukin-1beta (IL-1β), IL-6, IL-8 ,tumour necrosis factor-alpha (TNF-α), and transforming growth factorbeta-1 (TGF-β1), whereas others are associated with anti-inflammatory effects, such as IL-2, IL-4, IL-10, IL-12,IL-13, interferon-gamma (IFN-γ).

Molecular markers which are identified and characterized in saliva have distinct advantages in pathological diagnosis as they reflect the overall health and disease states in an individual. Because of the anatomical proximity of saliva to the oral cavity, salivary testing would be ideal for evaluating potentially malignant and malignant oral lesions[38],[55].

Several studies have assessed interleukin-6 (IL-6), a multifunctional cytokine that participates in the inflammatory and immune responses and has been shown to promote the growth of cancer cells as well as associated with an increased rate of metastasis and an altered immune status. IL-6 is an NF-κB dependent cytokine produced by inflammatory cells as well as tumor cells[56]. These cytokines are elevated as a result of localized production from at least two sources, either by the lesional epithelium itself or by the activated T lymphocytes present in the connective tissue affected with leukoplakia. Besides that, it is also known that oral epithelial cells are able to secrete IL-6 as a response to various microbial and chemical stimuli[57] .

Interleukin-6 first demonstrated as a marker for oral cancer [58]and subsequently in 2005 it was shown that salivary IL-6 had significant higher levels in OSCC compared to serum IL-6 [59]. In a study by Brailo et al, higher salivary IL-6 level was shown in oral leukoplakia compared to healthy subjeccts [60], similar results was also observed in the present study. In addition, compared to IL-6 level in OSCC, the IL-6 level in premalignent disorder was significantly lower. Similar observation was reported by Rhodus et al [25].

The potential value of IL-6 as a diagnostic marker of malignant transformation was considered keeping tobacco habits associated into consideration. A significant correlation has been demonstrated between the IL- 8 and IL-6 levels in

serum and saliva[61] . In another study,the elevated salivary IL-6 level in their observations was independent of any tobacco habit, unlike our findings where we noted a direct correlation between IL-6 and tobacco habits, implying that tobacco did play a significant role in IL-6 elevation. Significant differences in the concentration of salivary IL-6 between tobacco users and non users in both the study group as well as in control group have been demonstrated by Zhang et al.[62] who reported higher systemic level of IL-6 as a consequence of passive smoking in animal models. Erdemir et al.[63] on the other hand have reported no influence of smoking on IL-6 level in gingival crevicular fluid. The influence of tobacco on IL-6 is attributed to an interaction of the released tobacco products with keratinocytes[57] Nicotine from tobacco smoke and lipopolysaccharide from bacterial infection may also synergistically enhance IL-6 production in patients with periodontitis [64] .

In our study, among the premalignant lesions, we found that leukoplakia, OSF were associated with tobacco habits contributed to an elevated IL-6 level. Also showed increase in IL-6 level in OSF compared to other two premalignant lesions and control. Our observation thus confirms and extends the previous findings that IL-6 expression could be used as a specific marker for lesions that are at high risk of malignant transformation. The increased levels related to the clinical stage of the disease seemed to be the most sensitive parameter, have a better chance of identifying transformation to cancer particularly in the early stages.

CONCLUSION

Pro-inflammatory cytokines are elevated in the saliva of patients with PMD compared to controls, and thus suggesting that,

- Salivary IL-6 can be utilised as a potential biomarker for precancer that are at high risk of malignant transformation
- Patients with high levels of IL-6 levels may benefit from follow-up to have a better chance of identifying recurrences at an early stage.
- Validated diagnostic and/or prognostic significance which needs to be further confirmed by large population size at multicentre level.

Disclosures

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- Conflict of interest: The authors declare that they have no conflict of interests

BIBLIIOGRAPHIC REFERENCES

1. Lavanya R, Mamatha B, Waghray S, Chaitanya NCSK, Reddy MP, Bau DBG. Role of Tumor Markers in Oral Cancer: An Overview. J Adv Med Med Res. 2016 May 4;1–9.

2. Yardimci G, Kutlubay Z, Engin B, Tuzun Y. Precancerous lesions of oral mucosa. World J Clin Cases WJCC. 2014 Dec 16;2(12):866–72.

3. Vlková B, Stanko P, Minárik G, Tóthová Ľ, Szemes T, Baňasová L, et al. Salivary markers of oxidative stress in patients with oral premalignant lesions. Arch Oral Biol. 2012 Dec 1;57(12):1651–6.

4. Feller L, Lemmer J. Cell transformation and the evolution of a field of precancerization as it relates to oral leukoplakia. Int J Dent. 2011;2011.

5. Amagasa T, Yamashiro M, Uzawa N. Oral premalignant lesions: from a clinical perspective. Int J Clin Oncol. 2011;16(1):5–14.

6. Van der Waal I. Potentially malignant disorders of the oral and oropharyngeal mucosa; terminology, classification and present concepts of management. Oral Oncol. 2009;45(4–5):317–23.

7. van der Waal I. Potentially malignant disorders of the oral and oropharyngeal mucosa; present concepts of management. Oral Oncol. 2010;46(6):423–5.

8. Warnakulasuriya S, Johnson NW, Van der Waal I. Nomenclature and classification of potentially malignant disorders of the oral mucosa. J Oral Pathol Med. 2007;36(10):575–80.

9. van der Waal I, Reichart PA. Oral proliferative verrucous leukoplakia revisited. Oral Oncol. 2007;44(8):719–21.

10. Gupta PC, Mehta FS, Daftary DK, Pindborg JJ, Bhonsle RB, Jalnawalla PN, et al. Incidence rates of oral cancer and natural history of oral precancerous lesions in a 10-year follow-up study of Indian villagers. Community Dent Oral Epidemiol. 1980;8(6):283–333.

11. Bánóczy J, Csiba Á. Occurrence of epithelial dysplasia in oral leukoplakia: analysis and follow-up study of 12 cases. Oral Surg Oral Med Oral Pathol. 1976;42(6):766–74.

12. Eisen D, Carrozzo M, Bagan Sebastian J-V, Thongprasom K. Number V Oral lichen planus: clinical features and management. Oral Dis. 2005;11(6):338–49.

13. Gondivkar SM, Bhowate RR, Gadbail AR, Sarode SC, Gondivkar RS, Yuwanati M, et al. Quality of Life-related" Patient-reported Outcome Measures" in Oral Submucous Fibrosis Patients. J Contemp Dent Pract. 2018;19(3):331–8.

14. Krupaa RJ, Sankari SL, Masthan KMK, Rajesh E. Oral lichen planus: An overview. J Pharm Bioallied Sci. 2015 Apr;7(Suppl 1):S158–61.

15. Oral lichen planus: a retrospective study of 690 British patients - Ingafou - 2006 - Oral Diseases - Wiley Online Library [Internet]. [cited 2021 May 9]. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1601-0825.2005.01221.x

16. Pillai R, Balaram P, Reddiar KS. Pathogenesis of oral submucous fibrosis. Relationship to risk factors associated with oral cancer. Cancer. 1992;69(8):2011–20.

17. Jian XC, Liu SF, Shen ZH, Yang YH. Histomorphology of oral submucous fibrosis. Report of 24 cases. Chin Med J (Engl). 1988;101(7):505–9.

18. Hsue S-S, Wang W-C, Chen C-H, Lin C-C, Chen Y-K, Lin L-M. Malignant transformation in 1458 patients with potentially malignant oral mucosal disorders: a follow-up study based in a Taiwanese hospital. J Oral Pathol Med. 2007;36(1):25–9.

19. Pathogenesis of oral submucous fibrosis. Relationship to risk factors associated with oral cancer - Pillai - 1992 - Cancer - Wiley Online Library [Internet]. [cited 2021 May 9]. Available from: https://acsjournals.onlinelibrary.wiley.com/doi/abs/10.1002/1097-0142(19920415)69:8%3C2011::AID-CNCR2820690802%3E3.0.CO;2-B

20. Daftary DK. Oral precancerous lesions and conditions of tropical interest. Oral Dis Trop. 1993;402–28.

21. Sarode GS, Sarode SC, Maniyar N, Sharma N, Yerwadekar S, Patil S. Recent trends in predictive biomarkers for determining malignant potential of oral potentially malignant disorders. Oncol Rev [Internet]. 2019 Sep 10 [cited 2021 Feb 5];13(2). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6747023/

22. Singh P, Verma JK, Singh JK. Validation of Salivary Markers, IL-1β, IL-8 and Lgals3bp for Detection of Oral Squamous Cell Carcinoma in an Indian Population. Sci Rep. 2020 Apr 30;10(1):7365.

23. Tilakaratne WM, Sherriff M, Morgan PR, Odell EW. Grading oral epithelial dysplasia: analysis of individual features. J Oral Pathol Med. 2011;40(7):533–40.

24. Kanazawa T, Nishino H, Hasegawa M, Ohta Y, Iino Y, Ichimura K, et al. Interleukin-6 directly influences proliferation and invasion potential of head and neck cancer cells. Eur Arch Otorhinolaryngol. 2007;264(7):815–21.

25. Rhodus NL, Ho V, Miller CS, Myers S, Ondrey F. NF-κB dependent cytokine levels in saliva of patients with oral preneoplastic lesions and oral squamous cell carcinoma. Cancer Detect Prev. 2005;29(1):42–5.

26. Chen Z, Malhotra PS, Thomas GR, Ondrey FG, Duffey DC, Smith CW, et al. Expression of proinflammatory and proangiogenic cytokines in patients with head and neck cancer. Clin Cancer Res. 1999;5(6):1369–79.

27. Barak V, Selmi C, Schlesinger M, Blank M, Agmon-Levin N, Kalickman I, et al. Serum inflammatory cytokines, complement components, and soluble interleukin 2 receptor in primary biliary cirrhosis. J Autoimmun. 2009;33(3–4):178–82.

28. Kaur J, Jacobs R. Proinflammatory cytokine levels in oral lichen planus, oral leukoplakia, and oral submucous fibrosis. J Korean Assoc Oral Maxillofac Surg. 2015;41(4):171.

29. Mittal S, Bansal V, Garg SK, Atreja G, Bansal S. The diagnostic role of saliva: a review. Mittal Sanjeev Bansal Vikram Garg Shushant K Atreja Gaurav Bansal Sanjay Diagn Role

Saliva Rev En J Clin Exp Dent 2011 Vol 3 No 4 314-320 [Internet]. 2011 [cited 2021 Feb 4]; Available from: https://roderic.uv.es/handle/10550/54204

30. Zhang J-M, An J. Cytokines, Inflammation and Pain. Int Anesthesiol Clin. 2007;45(2):27–37.

31. Sahibzada HA, Khurshid Z, Khan RS, Naseem M, Siddique KM, Mali M, et al. Salivary IL-8, IL-6 and TNF-α as Potential Diagnostic Biomarkers for Oral Cancer. Diagnostics. 2017 Jun;7(2):21.

32. Inflammatory Cytokine Pattern Is Sex-Dependent in Mouse Cutaneous Melanoma Experimental Model [Internet]. [cited 2021 Apr 29]. Available from: https://www.hindawi.com/journals/jir/2017/9212134/

33. Gabay C. Interleukin-6 and chronic inflammation. Arthritis Res Ther. 2006 Jul 28;8(2):S3.

34. Márton IJ, Horváth J, Lábiscsák P, Márkus B, Dezső B, Szabó A, et al. Salivary IL-6 mRNA is a Robust Biomarker in Oral Squamous Cell Carcinoma. J Clin Med. 2019 Nov;8(11):1958.

35. Rhodus NL, Ho V, Miller CS, Myers S, Ondrey F. NF-κB dependent cytokine levels in saliva of patients with oral preneoplastic lesions and oral squamous cell carcinoma. Cancer Detect Prev. 2005 Jan 1;29(1):42–5.

36. Katakura A, Kamiyama I, Takano N, Shibahara T, Muramatsu T, Ishihara K, et al. Comparison of Salivary Cytokine Levels in Oral Cancer Patients and Healthy Subjects. Bull Tokyo Dent Coll. 2007;48(4):199–203.

37. Duffy SA, Taylor JMG, Terrell JE, Islam M, Li Y, Fowler KE, et al. Interleukin-6 predicts recurrence and survival among head and neck cancer patients. Cancer. 2008;113(4):750–7.

38. Salivary IL-6 levels in oral leukoplakia with dysplasia and its clinical relevance to tobacco habits and periodontitis | SpringerLink [Internet]. [cited 2021 Feb 4]. Available from: https://link.springer.com/article/10.1007/s00784-010-0435-5

39. Prasad G, McCullough M. Chemokines and Cytokines as Salivary Biomarkers for the Early Diagnosis of Oral Cancer. Int J Dent. 2013;2013:1–7.

40. Punyani SR, Sathawane RS. Salivary level of interleukin-8 in oral precancer and oral squamous cell carcinoma. Clin Oral Investig. 2013 Mar 1;17(2):517–24.

41. Czerninski R, Basile JR, Kartin-Gabay T, Laviv A, Barak V. Cytokines and tumor markers in potentially malignant disorders and oral squamous cell carcinoma: a pilot study. Oral Dis. 2014;20(5):477–81.

42. Abdel-Haq A, Kusnierz-Cabala B, Darczuk D, Sobuta E, Dumnicka P, Wojas-Pelc A, et al. Interleukin-6 and neopterin levels in the serum and saliva of patients with Lichen Planus and oral Lichen Planus. J Oral Pathol Med. 2014;43(10):734–9.

43. Rajkumar K, Nandhini G, Ramya R, Rajashree P, Kumar AR, Anandan SN. Validation of the diagnostic utility of salivary interleukin 8 in the differentiation of potentially malignant oral lesions and oral squamous cell carcinoma in a region with high endemicity. Oral Surg Oral Med Oral Pathol Oral Radiol. 2014 Sep 1;118(3):309–19.

44. Selvam NP, Sadaksharam J. Salivary interleukin-6 in the detection of oral cancer and precancer. Asia Pac J Clin Oncol. 2015;11(3):236–41.

45. Dineshkumar T, Ashwini BK, Rameshkumar A, Rajashree P, Ramya R, Rajkumar K. Salivary and Serum Interleukin-6 Levels in Oral Premalignant Disorders and Squamous Cell Carcinoma: Diagnostic Value and Clinicopathologic Correlations. Asian Pac J Cancer Prev APJCP. 2016;17(11):4899–906.

46. Bagan L, Sáez GT, Tormos MC, Labaig-Rueda C, Murillo-Cortes J, Bagan JV. Salivary and serum interleukin-6 levels in proliferative verrucous leukoplakia. Clin Oral Investig. 2016 May 1;20(4):737–43.

47. Qin R, Steel A, Fazel N. Oral mucosa biology and salivary biomarkers. Clin Dermatol. 2017 Sep 1;35(5):477–83.

48. Khurshid Z, Zafar MS, Khan RS, Najeeb S, Slowey PD, Rehman IU. Chapter Two - Role of Salivary Biomarkers in Oral Cancer Detection. In: Makowski GS, editor. Advances in Clinical Chemistry [Internet]. Elsevier; 2018 [cited 2021 Feb 2]. p. 23–70. Available from: http://www.sciencedirect.com/science/article/pii/S0065242318300313

49. Mozaffari HR, Sharifi R, Mirbahari S, Montazerian S, Sadeghi M, Rostami S. A systematic review and meta-analysis study of salivary and serum interleukin-8 levels in oral lichen planus. Adv Dermatol Allergol Dermatol Alergol. 2018 Dec;35(6):599–604.

50. Mozaffari HR, Sharifi R, Sadeghi M. Interleukin-6 levels in the serum and saliva of patients with oral lichen planus compared with healthy controls: a meta-analysis study. Cent-Eur J Immunol. 2018;43(1):103–8.

51. Gug IT, Tertis M, Hosu O, Cristea C. Salivary biomarkers detection: Analytical and immunological methods overview. TrAC Trends Anal Chem. 2019 Apr 1;113:301–16.

52. Babiuch K, Kuśnierz-Cabala B, Kęsek B, Okoń K, Darczuk D, Chomyszyn-Gajewska M. Evaluation of Proinflammatory, NF-kappaB Dependent Cytokines: IL-1α, IL-6, IL-8, and TNF-α in Tissue Specimens and Saliva of Patients with Oral Squamous Cell Carcinoma and Oral Potentially Malignant Disorders. J Clin Med. 2020 Mar;9(3):867.

53. Martina E, Campanati A, Diotallevi F, Offidani A. Saliva and Oral Diseases. J Clin Med. 2020 Feb;9(2):466.

54. Roi A, Roi CI, Negruțiu ML, Riviș M, Sinescu C, Rusu L-C. The Challenges of OSCC Diagnosis: Salivary Cytokines as Potential Biomarkers. J Clin Med. 2020 Sep;9(9):2866.

55. Ferguson DB. Current diagnostic uses of saliva. J Dent Res. 1987;66(2):420–4.

56. Wang P-L, Ohura K, Fujii T, Oido-Mori M, Kowashi Y, Kikuchi M, et al. DNA microarray analysis of human gingival fibroblasts from healthy and inflammatory gingival tissues. Biochem Biophys Res Commun. 2003;305(4):970–3.

57. Chang M-C, Wu H-L, Lee J-J, Lee P-H, Chang H-H, Hahn L-J, et al. The induction of prostaglandin E2 production, interleukin-6 production, cell cycle arrest, and cytotoxicity in primary oral keratinocytes and KB cancer cells by areca nut ingredients is differentially regulated by MEK/ERK activation. J Biol Chem. 2004;279(49):50676–83.

58. St. John MAR, Li Y, Zhou X, Denny P, Ho C-M, Montemagno C, et al. Interleukin 6 and Interleukin 8 as Potential Biomarkers for Oral Cavity and Oropharyngeal Squamous Cell Carcinoma. Arch Otolaryngol Neck Surg. 2004 Aug 1;130(8):929–35.

59. Cikes N, Lukać J, Virag M, Cekić-Arambasin A. Salivary and serum interleukin 6 and basic fibroblast growth factor levels in patients with oral squamous cell carcinoma. Minerva Stomatol. 2005;54(10):569–73.

60. Brailo V, Vučićević-Boras V, Cekić-Arambašin A, Alajbeg I, Milenović A, Lukač J. The significance of salivary interleukin 6 and tumor necrosis factor alpha in patients with oral leukoplakia. Oral Oncol. 2006;42(4):370–3.

61. John MAS, Li Y, Zhou X, Denny P, Ho C-M, Montemagno C, et al. Interleukin 6 and interleukin 8 as potential biomarkers for oral cavity and oropharyngeal squamous cell carcinoma. Arch Otolaryngol Neck Surg. 2004;130(8):929–35.

62. Zhang J, Liu Y, Shi J, Larson DF, Watson RR. Side-stream cigarette smoke induces dose–response in systemic inflammatory cytokine production and oxidative stress. Exp Biol Med. 2002;227(9):823–9.

63. Erdemir EO, Duran I, Haliloglu S. Effects of smoking on clinical parameters and the gingival crevicular fluid levels of IL-6 and TNF-α in patients with chronic periodontitis. J Clin Periodontol. 2004;31(2):99–104.

64. Wendell KJ, Stein SH. Regulation of cytokine production in human gingival fibroblasts following treatment with nicotine and lipopolysaccharide. J Periodontol. 2001;72(8):1038–44.

ANNEXURES

ANNEXURE 1

INFORMED CONSENT

I have been explained about the nature and purpose of the study in a language that I comprehend and I have fully understood all of it. I confirm that I had the opportunity to ask questions. I understand that my participation is voluntary and agree to cooperate with Dr. Ramsheena for the purpose of her study. I am also aware that this study/part of this study may be used for research and/or publication purposes. I agree to take part in this study

Signature

Place:

Date:

Name of participant:

Complete postal address:

This is to certify that the above consent has been obtained in my presence.

Witness 1 Witness 2

ANNEXURE II

A detailed history was recorded and clinical examination were carried out, and the characteristics of the different groups are described in Table **7**

Analysis of IL-6 in four different groups					
No.	**Age**	**Sex**	**Habit**	**Site of lesion**	**IL-6(pg/ml)**
Group 1	**Leukoplakia**				
1	57	M	Smoking	Buccal mucosa	10.2
2	61	M	Smoking and alcohol	FOM	15.3
3	65	M	Smoking and betel quid	Buccal mucosa	16.2
4	52	M	Smoking, alcohol and betel quid	Mandibular mucosa and sulcus	19.6
Group 2	**Lichen-planus**				
1	60	F	None	Buccal mucosa	15.4
2	57	F	None	Buccal mucosa	18.8
3	53	F	None	Buccal mucosa	13.7
4	49	F	None	Gingiva	12.1
Group 3	**OSMF**				
1	52	M	Smoking and betel quid	Buccal mucosa	18.4
2	48	M	Smoking, alcohol, betel quid	Buccal mucosa	31.2
3	63	M	Smoking, betel quid	Tounge	25.4
4	53	M	Smoking, betel quid	Buccal mucosa	21.6
Group 4	**Control**				
1	43	M	None	_	2.53
2	58	M	None	_	7.8
3	44	M	None	_	3.4
4	31	F	None	_	0.4
5	49	M	None	_	4.6
6	51	M	None	_	1.9
7	37	F	None	_	5.1
8	50	F	None	_	3.9
9	53	M	None	_	10.2
10	48	M	None	_	6.1
11	46	F	None	_	9.1
12	58	M	None	_	8.1