STUDY OF INTERLEUKIN 8 AND INTERLEUKIN 1B IN SALIVA OF PATIENTS WITH ORAL SQUAMOUS CELL CARCINOMA AND ORAL POTENTIALLY MALIGNANT DISORDERS

Dissertation submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY

In partial fulfillment for the Degree of

MASTER OF DENTAL SURGERY

BRANCH VI

ORAL PATHOLOGY AND MICROBIOLOGY

APRIL 2013

CERTIFICATE

This is to certify that this dissertation titled **"STUDY OF INTERLEUKIN 8 AND INTERLEUKIN 1B IN SALIVA OF PATIENTS WITH ORAL SQUAMOUS CELL CARCINOMA AND ORAL POTENTIALLY MALIGNANT DISORDERS"** is a bonafide dissertation performed by **AISWARYA LEKSHMY. S. U** under our guidance during the post graduate period 2010 – 2013.

This dissertation is submitted to **THE TAMILNADU DR. M. G. R. MEDICAL UNIVERSITY,** in partial fulfillment for the degree of **MASTER OF DENTAL SURGERY in ORAL PATHOLOGY AND MICROBIOLOGY, BRANCH VI.** It has not been submitted (partial or full) for the award of any other degree or diploma.

Dr. K. Ranganathan, M.D.S, M.S (Ohio) PhD
Professor and Head
Department of Oral & Maxillofacial Pathology
Ragas Dental College & Hospitals
Chennai

Dr. K. Ranganathan, MDS, MS(Ohio), Ph.D,
Professor and Head
Department of Oral & Maxillofacial Pathology
Ragas Dental College and Hospital, Chennai

Dr. Uma Devi K Rao, M.D.S.
Professor
Department of Oral & Maxillofacial Pathology
Ragas Dental College & Hospitals
Chennai

Dr. Umadevi K Rao, MDS
Professor
Department of Oral and Maxillofacial Pathology
Ragas Dental College and Hospital, Chennai

Dr Ramachandran S, M.D.S.
Principal
Ragas Dental College, Chennai

PRINCIPAL
RAGAS DENTAL COLLEGE & HOSPITAL
CHENNAI

Acknowledgement

ACKNOWLEDGEMENT

"Gratitude is not only the greatest of virtues, but the parent of all others."

— Marcus Tullius Cicero

I express my heartfelt gratitude to my guide and mentor, **Dr Ranganathan K**, MDS, MS(Ohio), PhD, Professor and Head, Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital, for his exemplary guidance, patient listening, constant encouragement throughout the course of this thesis and for having faith in me without which this could never have been possible.

I am deeply grateful to **Dr Uma Devi K Rao,** MDS, Professor, Department of Oral and Maxillofacial Pathology, Ragas Dental College & Hospital for her priceless support, valuable advice and guidance.

I am immensely grateful to **Dr Elizabeth Joshua**, MDS, Professor, Department of Oral and Maxillofacial Pathology, Ragas Dental College & Hospital for her cordial support and encouragement.

My sincere gratitude to **Dr Rooban**, MDS, Professor, Department of Oral and Maxillofacial Pathology, Ragas Dental College & Hospital for his encouragement and help.

I thank **Dr. S. Ramachandran, Principal, and Mr. Kanagaraj,** Chairman Ragas Dental College & Hospital for their permission to use the amenities of the institution.

I express my gratitude to **Dr K M Vidya, Reader,** Department of Oral and Maxillofacial Pathology, Ragas Dental College & Hospital for her encouragement, help and constant support all through the years of my study. I thank **Dr Lavanya N,**

Reade**r** and **Dr Lavanya C,** senior lecturer**,** Department of Oral and Maxillofacial Pathology, Ragas Dental College & Hospital, for their help and support.

I thank our Research Assistant **Mrs. Kavitha Wilson,** Biostatistician **Mrs. Deepa** and Lab Technician **Mr. Rajan** for their help and encouragement throughout my postgraduate course.

I express my immense gratitude to **Dr Rajaraman R**, Surgical Oncologist, for helping me with sample collection and being extremely supportive throughout the period of my dissertation.

I thank **Dr Praveen B** for his valuable support and help.

I am sincerely grateful to **DR C. S. Mani**, Surgical Oncologist, Kumaran Hospital for his help and support in my dissertation. I am also grateful to **Dr Shivakumar**, MD, Kumaran Hospital.

I thank **Dr Madan Mohan,** Professor and Head, Department of Oral and maxillofacial surgery, Karpagavinayaka Institute of Dental Sciences for his immense support and help in sample collection.

I am thankful to **Dr Veerabahu**, **MDS,** Professor and Head of the Department of Oral and Maxillofacial Pathology for being supportive and helping in sample collection.

I express my sincere gratitude to **Dr K V Arun**, Professor, Department of Periodontics for his valuble advices, constant support and help through all the stages of my dissertation. I thank **Dr Swarna Alamelu,** Department of Periodontics for her encouragement and support.

I am deeply grateful to **Dr Ramesh I. S**, Lab director, **Mrs Padmaasree R**, Consultant microbiologist and **Dr Pukalendi V**, Senior consultant, Medall lab,

Voluntary Health Services, for their selfless guidance, support and help in my dissertation. I thank **Mr Vinodh**, Lab technician, Medall lab, for helping me patiently in running the ELISA experiment.

I thank **Dr Ravanan D Ramanujam** for heping me in the statistical analysis.

I am extremely thankful to my batchmates **Jaishlal M. S, Nithya S, Shanmughapriya P, Sudharsan R and Vaishnavi S** for being with me in all ups and downs. I thank my **Seniors and Juniors** for all their encouragement and support, specially, **Sravanthi** for helping me in sample collection.

Words are not enough to thank my loving family, my husband **Midhun D. S**, my parents **Mr G. Umeswaran Nair** and **Mrs Latha. U. Nair**, my brother Mr **Narendra Nath S. U** and my mother in law **Mrs Devaki Amma** for having faith in me, giving me care and strength to endure every hardship in my life and for their support which helped me in completing this task through various stages.

I am thankful to **Vasanthi akka**, who was always there to help. I also thank all the **nursing staffs** who helped me in sample collection.

I sincerely thank all the **patients** who participated in my dissertation, without whom this study would not have been completed.

Above all I thank **God almighty** for making everything possible. I thank him for bringing all the above mentioned people to play a role in my life, to make my dissertation a reality and making me realise the beauty of being grateful.

"Sarve bhavantu sukhina Sarve santu nirāmayā
sarve bhadrāni paśyantu Mā kaścit dukha bhāgbhavet"

CONTENTS

ABSTRACT

Background: Oral cancer is a fatal disease with a bad prognosis. It is often preceded by oral potentially malignant disorders (OPMD). Cytokines Interleukin-8 (IL8) and Interleukin-1Beta (IL1B) are produced in a dysregulated fashion in oral cancer. Salivary levels of these cytokines may be early markers of malignant transformation of OPMD.

Aim and Objective: To study the levels of Interleukin-8 and Interleukin1B in saliva of patients with oral squamous cell carcinoma (OSCC), oral potentially malignant disorders and normal controls.

Materials and methods: Saliva from 18 subjects with OSCC, 16 subjects with Oral submucous fibrosis (OSF), 14 subjects with Leukoplakia (Dysplasia) and 24 normal patients constituted the study group. Salivary IL 8 and IL1B were analysed using solid phase sandwich Enzyme Linked Immunosorbant Assay (ELISA) technique.

Results: Levels of salivary IL 8 and IL1B were significantly elevated in OSCC when compared to controls. OPMD showed no significant difference when compared to controls.

Conclusion: Salivary levels of IL 8 and IL 1B are higher in OSCC than in OPMD and normal. The levels in OPMD and normal were similar. Based on our results, in a limited sample, the cytokine IL 8 is useful in identifying transformation of OPMD. IL 1B shows potential in leukoplakia.

Keywords: Saliva, Biomarker, Cytokines, Interleukin 8, Interleukin IB, Oral squamous cell carcinoma.

Introduction

Cancer is the leading cause of death in both economically developed countries and developing countries.[1] Globally 12.7 million cancer cases and 7.6 million cancer deaths occurred in 2008.[2] Of this oral cavity cancer (including lip cancer) accounted for 263,900 new cases and 128,000 deaths. In India out of 5,56,400 cancer deaths in 2010, 45000 cases were due to oral cancer which makes it one of the most fatal disease in the country. Among 84000 tobacco related cancers there were twice as many deaths from oral cancers than lung cancers.[3, 4]

More than 95% of the carcinomas of the oral cavity are squamous cell carcinomas. Carcinoma of oral cavity in males, in developing countries, is the sixth commonest cancer after lung, prostrate, colorectal, stomach and bladder cancer, while in females; it is the tenth most common site of cancer after breast, colorectal, lung, stomach, uterus, cervix, ovary, bladder and liver.[5]

Studies including a recent study in 2012 reaffirmed that oral cancer forms a major part of the cancer load in parts of India.[3,6] Tobacco and arecanut are the two most important known risk factors for the development of oral cancer.[7,8] Cofactors in development of Oral Squamous Cell Carcinoma (OSCC) include dietary factors, immunodeficiency and viral infections like HPV 16/18[6].

Though the oral cavity is very accessible to routine examination oral cancer has bad prognosis because it is usually diagnosed in late stages. OSCC is often preceded by Oral Potentially Malignant Disorders (OPMD) such as leukoplakia, erythroplakia, erosive lichen planus and Oral Submucous Fibrosis (OSF). The malignant conversion of OPMD can to some extent be predicted by histopatholgical findings. Moderate dysplasia has 4-11% chances of malignant transformation and severe dysplasia has 20-35% chances of malignant transformation.[9] Surrogate biomarkers help in identifying

the lesions that carry a higher risk of malignant transformation. Salivary biomarkers are gaining importance because of their ease of accessibility, patient compliance and advances in technique that enable identification of specific markers.[9,10, 11, 12]

Salivary levels of Interleukin 8 (IL8) and Interleukin I Beta (IL1B) are known to be elevated in oral cancer.[10, 11] They are chemokines that are deregulated in oral squamous cell carcinoma. Chemokines regulate growth, trafficking, signalling, differentiation of stromal and tumor cells, affect the tumor microenvironment and influence the behaviour of malignant cells[12]

Elevated IL-1B concentrations have been identified in tumors of breast, colon, lung, head and neck cancers, and melanomas with poor prognoses.[13.14.15,16,17] IL1B promotes tumor proliferation, angiogenesis, and metastases through autocrine and paracrine effects within the tumor and in the microenvironment.[18] While the exact mechanisms by which IL-1B promotes tumor growth remain unclear the protein is believed to act by induction of pro-metastatic genes such as matrix metalloproteinases and through paracrine stimulation angiogenic proteins and growth factors such as VEGF, IL-8, IL-6, TNFalpha, and TGF. [19]

IL 8 is a pro-inflammatory chemokine which exerts multiple effects on different cell types present in the tumor and its microenvironment. Tumor derived IL 8 can increase the proliferative activity of tumor cells, enhance the survival and promote angiogenesis by inducing the endothelial cells. Moreover, it can also activate the tumor-associated macrophages which lead to the secretion of additional growth factors. These growth factors further increase the rate of cell proliferation and invasion.[20]

Saliva as a diagnostic tool has the potential to identify altered levels of cytokines in oral malignant and potentially malignant disorders.[9,10.46] The purpose of this study was to study the levels of IL8 and IL1B in saliva in patients with oral malignant and potentially malignant disorders and ascertain their relevance in OSCC and OPMD.

Aims and Objectives

AIM:

To detect the levels of IL8 and IL1B in saliva of patients with OSCC, OPMD and control group (Normal).

OBJECTIVES:

1. To study the levels of IL8 and IL1B in saliva of patients with OSCC by using solid phase sandwich ELISA with biotinylated IL8 and IL1B antibodies (Diaclone^{TM}France)(Annexure I and II).
2. To study the levels of IL8 and IL1B in saliva of patients with OPMD (oral submucous fibrosis and leukoplakia) by using solid phase sandwich ELISA with biotinylated IL8 and IL1B antibodies (Diaclone^{TM}France).
3. To study the levels of IL8 and IL1B in saliva of normal controls including patients with periodontitis and patients without periodontitis by using solid phase sandwich ELISA with biotinylated IL8 and IL1B antibodies (Diaclone^{TM}France).

NULL HYPOTHESIS:

IL8 and IL1B levels are similar in saliva of patients with OSCC, OPMD (Oral submucous fibrosis and Leukoplakia) and controls (Normal).

Materials and Methods

METHODOLOGY

Institutional Review Board (IRB) approval was obtained(Annexure III) for the given project. All samples were collected after obtaining consent from the subjects. (Annexure IV).

Groups:

Group I - Oral squamous cell carcinoma patients, n=18

Group II - Oral potentially malignant disorders, n=30

- Group IIA - Oral submucous fibrosis, n=16
- Group IIB - Leukoplakia, n=14

Group III - Normal controls, n=24

- Group IIIA - With periodontitis, n=12
- Group IIIB - Without periodontitis, n=12

DIAGNOSTIC CRTERIA

OSCC - Histologically diagnosed Squamous cell carcinoma of oral cavity (excluding lip).

OSF - Oral submucous fibrosis which is an insidious, chronic disease affecting any part of the oral cavity and sometimes the pharynx. Occasionally it is preceded by and/or associated with vesicle formation and is always associated with a juxtaepithelial inflammatory reaction followed by progressive hyalinization of the lamina propria.

Leukoplakia - Those with Epithelial dysplasia in clinically diagnosed Leukoplakia should be used to recognize white plaques of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer.

Inclusion criteria for group I subjects:

- Patients with Squamous cell carcinoma of oral cavity in the following sites : Buccal mucosa, tongue, gingiva, alveolus, hard and soft palate.
- Patients should not have undergone any treatments (chemotherapy, radiotherapy, surgery or any alternative remedies)

Inclusion criteria for group II subjects:

- Patients with Oral potentially malignant disorders (Oral submucous fibrosis and Leukoplakia with histologically diagnosed epithelial dysplasia)

Exclusion criteria for group I and II subjects:

- Any prior malignancy
- Fever at present or for the past one month
- Immunodeficiency – immune-suppression in transplant patients, HIV infection
- Autoimmune disorders(arthritis, psoriasis, inflammatory bowel disease, Sjogren's syndrome)
- Hepatitis virus infection

Inclusion criteria for group III subjects:

Subjects without periodontitis

- Healthy, age and gender matched subjects.
- Subjects without periodontitis and other metabolic and systemic diseases.

Exclusion criteria for group III subjects

- Subjects with any pathology of oral mucosa.

Criteria used for assessing Periodontitis[21]

1. Plaque scores more than 30% of sites
2. Bleeding scores more than 30% of sites
3. Pocket depth greater than or equal to 3mm
4. Clinical attachment loss (CAL) greater than or equal to 2mm

Pocket depth is measured from the gingival margin to the base of the sulccus and clinical attachment loss is measured from the cement-enamel junction to the base of the pocket. All the parameters are measured fron six sites per tooth – mesio-buccal, mid-buccal, distobuccal, mesio-lingual, mid-lingual and disto-lingual.

METHOD OF COLLECTING SALIVA: [22]

Unstimulated whole saliva was collected at a specific time interval, between 9 to 12 am either before breakfast or one hour after having breakfast. Subject is seated in a semi reclined comfortable position.

- The subjects were asked to avoid brushing teeth within 1 hour prior to collection, using salivary stimulants: chewing gum, lemon drops, granulated sugar, consuming a major meal within 1 hour prior to collection and consuming acidic or high sugar foods within 20 minutes prior to collection.
- The subjects were given drinking water (bottled) and asked to rinse their mouth out well (without drinking the water).
- Five minutes after this oral rinse, the subjects were asked to spit whole saliva (WS) every minute for 5 to10 minutes without swallowing to the sterile container.

- Subjects were advised to refrain from talking, drop down the head and let the saliva run naturally to the front of the mouth, hold for a while and spit into the sterile container provided about once a minute for up to 10 minutes.
- Subjects were advised not to cough up mucus or vomit. (Samples diluted with mucus or vomits were discarded).
- Collected samples were labelled with a sample identification number and placed on ice for transport.
- The case history and all medications taken and prescribed were recorded with time & date of sample collection.

Storage of the samples:

1. Collected samples are centrifuged at -10 to -20 degree centigrade at 3500rpm for 15- 20mts.
2. The supernatant and the sediment is stored separately, the sediment (pellet) is kept in the centrifuged tube and marked.
3. The supernatant is aliquoted into separate micro centrifuge tubes (eppendorf) by pippeting approximately 1.5ml into each tube.
4. The supernatant and pellet are stored at -70 degree Celsius till processing.

EQUIPMENTS AND CHEMICAL REAGENTS NEEDED:

1. Oral examination devices

   Mouth mirror, Williams probe, disposable gloves and mask
2. Elisa kit for IL8 and IL1B
3. Elisa reader
4. Incubator
5. Autoclave
6. 10, 50, 100, and 200 µl micropipettes and disposable pipette tips
7. Timer
8. Disposable vials and troughs
9. Deionized water
10. Absorbent tissue
11. Negative 70 degree centigrade ultra low temperature storage cabinet
12. Labels
13. PBS
14. Polypropylene conical tube
15. Centrifuge Tubes
16. Laboratory Centrifuge
17. Sterilized containers for saliva collection
18. Ice pack (for transfer)

PROCEDURE:

Enzyme linked Immunosorbant Assay (ELISA)

- Solid phase sandwich ELISA for IL8 and IL1B was used for quantitative determination of salivary IL8 and IL1B levels respectively. (Manufacturer – Diaclone^{TM})

PRINCIPLE OF THE ASSAY

- The wells of the microtitre strip plate have been coated with a capture antibody highly specific for IL8 and IL-1B provided during manufacture.
- Binding of IL8 and IL1B in samples and known standards to the capture antibodies and subsequent binding of the biotinylated anti-IL8 and IL-1B secondary antibody to the analyte is completed during the same incubation period. Any excess unbound analyte and secondary antibody was removed.
- The HRP conjugate solution is then added to every well including the zero wells, following incubation excess conjugate is removed by careful washing.
- A chromogen substrate is added to the wells resulting in the progressive development of a blue coloured complex with the conjugate.
- The colour development is then stopped by the addition of acid turning the resultant final product yellow.
- The intensity of the produced coloured complex is directly proportional to the concentration of IL8 and IL-1B present in the samples and standards.
- The absorbance of the colour complex is then measured and the generated values for each standard are plotted against expected concentration forming a standard curve.(Annexure V)

- The absorbance value is measured on a spectrophotometer using a wavelength of 450 nm.
- The standard curve was then used to determine the concentration of IL 8 and IL-1B in the samples tested.

PREPARATION OF REAGENTS

- The kit was stored at 2-8^{0}C after procurement and all the reagents were brought to room temperature before used.
- Preparation of Wash Buffer was done by diluting the wash buffer concentrate 200 fold with distilled water to give a 1x working solution. Entire contents (10 ml) of the Washing Buffer Concentrate was poured into into a clean 2,000 ml graduated cylinder and final volume was made to 2,000 ml with glass-distilled or deionized water.
- Preparation of Standard Diluent Buffer was done by adding the contents of the vial (10x concentrate) to 225ml of distilled water before use.
- Standard was prepared by reconstituting the standard vials using standard diluents for serum and plasma
- This reconstitution gives a stock solution of 2000pg/ml of IL-8 and 500pg/ml of IL 1B.
- Control was prepared by reconstituting freeze-dried control vials with the Standard Diluent for serum and plasma.
- Preparation of Biotinylated anti-IL-8 was done immediately before use by diluting the biotinylated anti-IL-8 with the biotinylated antibody diluent in a clean glass vial using volumes appropriate to the number of required wells.

- Streptavidin-HRP was prepared by diluting the 5µl vial with 0.5ml of HRP diluent immediately before use.

SERIAL DILUTION OF STANDARD

- Reconstituted standard was mixed gently by repeated aspirations/ejections.
- Serial dilutions of the standard are made directly in the assay plate to provide the concentration range from 2000 to 62.5pg/ml.
- Immediately after reconstitution 200µl of the reconstituted standard was added to wells A1 and A2, which provides the highest concentration standard at 2000pg/ml
- 100µl of appropriate standard diluent was added to the remaining standard wells B1 and B2 to F1 and F2
- 100µl from wells A1 and A2 was aspirated, added to B1 and B2 and mixed by repeated aspirations and ejections taking care not to scratch the inner surface of the wells
- This 1:1 dilution was continued by using 100µl from wells B1 and B2 through to wells F1 and F2 providing a serial diluted standard curve ranging from 2000pg/ml to 62.5pg/ml for IL 8 and from 500pg/ml to15.6pg/ml of IL 1B.

FLOWCHART OF EXPERIMENT

Add 100 µl of sample and diluted standard/controls and 50µl Biotinylated anti-IL-8

↓

Incubate 1 hours at room temperature for IL 8 and 3 hours for IL 1B

↓

Wash three times

↓

Add 100µl of Streptavidin-HRP

↓

Incubate 30min at room temperature

↓

Wash three times

↓

Add 100µl of ready-to-use TMB. Let the color develop for 12-15 mn.

↓

Add 100µl H_2SO_4

↓

Read Absorbance at 450 nm

STATISTICS

Sample Size

The sample size of 30 in each group has been determined from the Standard Statistical Table (Expecting a large effect size of 0.4 to detect the minimum difference between control and study groups under an approximate power of 0.9 with the level of significance of 0.05).

The allotted study period was 10 months. Only 72 cases satisfying the criteria was obtained in the time period of the study and hence the sample size was reduced as shown below.

TYPE OF SAMPLE	SAMPLE SIZE
Oral squamous cell carcinoma	18
Oral submucous fibrosis	16
Leukoplakia	14
Controls with periodontitis	12
Controls without periodontitis	12

Statistical Analysis

Data entry and analysis was done using SPSS (Statistical Package of Social Science™) version 17. The differences in means between more than one group were assessed using Analysis Of Variance (ANOVA)test and multiple comparisons were done using Tukey HSD test. P value of < 0.05 was considered to be statistically significant.

Review of Literature

Cancer is a potentially fatal disease affecting a significant part of the population and one of the leading causes of death worldwide. In India, the International Agency for Research on Cancer estimated indirectly that about 635000 people died from cancer in 2008, representing about 8% of all estimated global cancer deaths and about 6% of all deaths in India.[3]

Oral cancer is a serious debilitating disease and a growing problem in India as well as in many parts of the world. Oral and pharyngeal cancer, grouped together, is the sixth most common cancer in the world. [23]

ORAL SQUAMOUS CELL CARCINOMA

Oral cancer remains a lethal disease for over 50% of cases diagnosed annually[24]. Smoking, smokeless tobacco products, alcohol use, arecanut and HPV infections are the major risk factors for oral cavity cancer, with smoking and alcohol having synergistic effects. The contribution of each of these risk factors to the burden varies across regions. Accordingly there is a significant difference in the incidence of oral cancer in different regions of the world. The age-adjusted rates of oral cancer vary from over 20 per 100,000 populations in India, to 10 per 100,000 in the U.S., and less than 2 per 100,000 in the Middle East.[17] Smoking accounts for 42% of deaths from cancers of the oral cavity (including the pharynx) and heavy alcohol consumption for 16% of the deaths worldwide. In high-income countries, smoking and alcohol consumption accounts for 70% and 30% of cancer deaths, respectively. At the same time in India, Taiwan, and other neighbouring countries smokeless tobacco products, arecanut and betel quid with or without tobacco are the major risk factors for oral cavity cancer. [7]

The global burden of cancer continues to increase largely because of the aging and growth of the world population and an increasing adoption of cancer-causing behaviours, particularly smoking, within economically developing countries. More than 95% of the carcinomas of the oral cavity are of squamous cell type, in nature. They constitute a major health problem in developing countries, representing a leading cause of death. The survival index continues to be small (50%), as compared to the progress in diagnosis and treatment of other malignant tumors. In India, 60-80% of patients present with advanced disease as compared to 40% in developed countries. [6]

This is reflected by the fact that despite easy accessibility of the oral cavity for regular examination most cases are in advanced stages at the time of detection and the survival rate for oral cancer has remained essentially unchanged over the past three decades. Detecting oral cancer in early stages, when these are amendable to single modality therapies, offers the best chance of long term survival.[25]

ORAL SUBMUCOUS FIBROSIS

Oral submucous fibrosis (OSF) is an insidious, chronic disease affecting any part of the oral cavity and sometimes the pharynx. Occasionally it is preceded by and/or associated with vesicle formation and is always associated with a juxtaepithelial inflammatory reaction followed by progressive hyalinization of the lamina propria. Different populations may show different sites of involvement within the mouth.[26] The etiology of OSMF is mainly attribauted to the use of areca nut predominantly found in India. Other etiologic factors such as chillies, lime, tobacco, nutritional deficiencies such as iron and zinc, immunological disorders, and collagen disorders also have been suggested.[27]

Commercially available products such as pan masala, betel quid, guthka and mawa have high concentrates of areca which interferes with the molecular processes of deposition and/or degradation of extracellular matrix molecules such as collagen. The habit of chewing pan masala has been found to be associated with OSMF more than betel quid chewing. Direct correlation was seen in occurrence of OSMF with frequency rather than duration of chewing.[28] The early and late forms of presentation outlined in OSMF are well recognized as a potentially malignant disorder. [29] OSMF has a high rate of morbidity because it causes a progressive inability to open the mouth, resulting in difficulty in eating and consequent nutritional deficiencies. OSMF also has a significant mortality rate as it has potential to transform into oral cancer, particularly squamous cell carcinoma, at a rate of 7.6%.[29] Early detection and management of OSMF can significantly reduce the mortality and morbidity associated with it.[30]

DYSPLASIA

Many oral cancers pass through a premalignant phase (dysplasia or carcinoma in situ), whereas others appear to arise de novo without clinical or microscopic evidence of a pre-existing lesion. This includes leukoplakia, erythroplakia, oral submucous fibrosis, actinic keratosis, erosive lichen planus etc.[31,32] At present, biopsy is mandatory considering the malignant potential of these lesions. Histopathological examination of haematoxylin and eosin stained sections for the presence of epithelial dysplasia is currently the gold standard for assessment of potentially malignant disorders.

'The term leukoplakia should be used to recognize white plaques of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer'. [31] About 50% of biopsied leukoplakias show dysplasia and overall the rate of malignant transformation in leukoplakia is only about 0.1 - 2% per year. Higher rates of about 20% have been reported in non-homogeneous lesions which are more likely to show dysplasia on biopsy.[33] The malignat conversion of precancerous lesions and conditions can to some extent predicted by histopatholgical findings: Moderate dysplasia has 4-11% chances of malignant transformation and severe dyplasia has 20-35% chances of malignant transformation.[7]

BIOMARKERS

Hulka and colleagues defined biomarker as "Cellular, biochemical or molecular alterations that are measurable in biological media such as human tissues, cells, or fluids."[34] These are biological characteristics that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention. In practice, biomarkers include tools and technologies that can aid in understanding the prediction, cause, diagnosis, progression, regression, or outcome of treatment of disease.[35] Many biomarkers has been studied and validated in serum and saliva for various oral and systemic diseases.

SALIVA

Saliva is a clear, slightly acidic and complex biological fluid composed of secretions from major salivary glands: the parotid, submandibular, and sublingual

glands, as well as multitudes of minor glands including labial, buccal, lingual, and palatal tissues. In general, human salivary glands produce about 1–1.5 L of serous and mucinous saliva daily by combining water, salts, and an abundance of molecules from the blood with a cocktail of salivary proteins in the oral cavity to give rise to the multi-constituent whole saliva. The pH of saliva ranges from 6.0–7.0.[36]

CHEMICAL COMPOSITION AND FUNCTIONS OF SALIVA

Water is the major constituent of saliva (97%), besides water salivary fluid contains proteins, post-translationally modified proteins (e.g., glycoproteins, phosphoproteins), peptides, lipids, minerals, and other small compounds. Upon release of glandular secretions into the oral cavity, the fluid is mixed with a variety of exocrine, nonexocrine, cellular, and exogeneous components to ultimately form whole saliva (WS). Whole saliva is a complex fluid which consists of salivary secretions from different salivary glands, sloughed oral epithelial cells, nasopharyngeal discharge, food debris, gingival crevicular fluid, bacteria and their products.[37]

Saliva has several types of functions that are of profound importance for the oral health.[38] It include digestion, antimicrobial activity, buffering capacity, lubrication cleansing action etc. All these function are carried out by the proteins, enzymes and other compounds present in saliva (Annexure VI). Saliva is a complex fluid containing a variety of enzymes, hormones, antibodies, inflammatory markers, antimicrobial constituents, and growth factors. Many of these enter saliva from the blood by passing through the spaces between cells by transcellular or paracellular routes. Therefore, most compounds found in blood are also present in saliva, thus

saliva is functionally equivalent to serum in reflecting the physiological and disease states of the body.

SALIVA PHYSIOLOGY

Oral fluid is primarily composed of water (97%) electrolytes, immunoglobins, enzymes and proteins.[39] The salivary glands are composed of specialized epithelial cells which are of two types: the acinar and ductal cells. The fluid is generated and most of the protein synthesis and secretion takes place in the acinar region. Amino acids enter the acinar cells by means of active transport, and after intracellular protein synthesis, the majority of proteins are stored in storage granules that are released in response to secretory stimulation. The initial fluid is isotonic in nature and is derived from the local vasculature. While acinar cells are water-permeable, ductal cells are not. The ductal cells actively absorb most of the Na^+ and Cl^- ions from the primary salivary secretion and secrete small amounts of K^+ and HCO3 and some proteins. The primary salivary secretion is thus modified, and the final salivary secretion as it enters the oral cavity is hypotonic.[37]

Salivary secretions are classified as serous, mucous, or mixed. Serous secretions contain more water than mucous. Different salivary glands produce saliva with a characteristic protein composition. When salivary flow is unstimulated, such as in resting saliva; the submandibular contributes a major percentage to whole saliva volume followed by parotid, sublingual and minor salivary glands respectively (Annexure VI). Although the minor salivary glands are not major contributors to the whole-saliva volume, they play a significant role in the lubrication and protection of the oral mucosa because of their mucous secretions. Parotid gland secretion is purely

serous, and submandibular and sublingual gland secretions are mixed (mucous and serous).

The primary saliva, formed by the acinar cells, has an ionic composition similar to that of plasma. It is modified by the ductal cells to a hypotonic solution by reabsorption of sodium and chloride without water.[37.] The flow rate of resting saliva for all three glands is very low, approximately one-tenth of that during stimulated flow. The total amount of saliva secreted varies among individuals and environmental factors.[39] Stimulation affects the quantity of saliva, the concentrations of some constituents and the pH of the fluid[40].

Salivary flow rate, which can increase to 10 ml/min when stimulated, influences oral fluid pH. The bicarbonate content of oral fluid increases with stimulation. Circadian rhythms, physical activity and health status of the individual also influence physiology and flow rate of oral fluid.[41]

SALIVA AS A DIAGNOSTIC FLUID

Saliva is a clinically informative, biological fluid that is useful for novel approaches to prognosis, laboratory or clinical diagnosis, and monitoring and management of patients with both oral and systemic diseases. Saliva can reflect the physiologic state of the body, including emotional, endocrinal, nutritional and metabolic variations and acts as a source for the monitoring of oral as well as systemic health. The application of saliva has dramatically got expanded due to the technological advances for the past two decades. The achievements of high-throughput approaches afforded by biotechnology and nanotechnology allow for disease-specific salivary biomarker discovery and establishment of rapid, multiplex, and miniaturized

analytical assays. The avenue of saliva diagnostics incorporating transcriptomic, proteomic and metabolomic findings will enable the use of salivary biomarkers to diagnose a disease state and to monitor the progression remission and reoccurrence of diseases and therapeutic outcomes. [42] .Salivary diagnostics is an emerging field that has tremendous potential in clinical applications because its collection is non-invasive and it contains a wide spectrum of analytes which can serve as biomarkers for assessment of oral and systemic health.

Saliva clearly meets the demands for an inexpensive, noninvasive, and easy-to-use screening method. As a diagnostic specimen in the clinic, saliva has many advantages in terms of collection, storage, shipping, and voluminous sampling; all of these processes can be carried out very economically compared with serum or urine. Saliva is also easier to handle during diagnostic procedures than blood because it does not clot, thus reducing the number of manipulations required. Moreover, for healthcare professionals, a salivary test is safer than using serum, which is more likely to expose operators to blood-borne diseases. For the patients or examinees, the non-invasive collection approach could dramatically reduce anxiety and discomfort, and increase their willingness to undergo health inspections that will greatly increase the opportunity to monitor their general health over time and to diagnose morbidities in the early stage.[43,44,45,46] Composition of saliva is influenced directly or indirectly by oral and systemic diseases. Early detection, diagnosis and assessment of prognosis of various diseases could be possible by monitoring the composition of saliva. Many studies have been done to identify potential biomarkers in saliva for oral as well as systemic diseases.

The significance of salivary biomarkers in many oral diseases has been evaluated including periodontitis, oral cancer[10,11,] OPMD[46] and oral lichen planus.[47] None the less it has been also assessed in many systemic diseases like diabetes[48] cardiac diseases,[49] Sjogren syndrome[50,51] and infectious diseases like HIV[42]and Helicobacter pylori[52] infections. Moreover role of saliva in monitoring the levels of hormones and drugs have also been studied and evaluated. Point of care devices like Oral-AQ Oral Fluid Drugs of Abuse Rapid Test® for drug abuse and OraQuick® for HIV is commercially available.

COLLECTION OF SALIVA SAMPLE

The saliva sample can be collected in two ways: stimulated and unstimulated. Stimulated saliva can be collected after stimulation of salivary secretion using paraffin wax or citric acid. Unstimulated saliva is collected in the resting state. The submandibular glands are the major contributors to resting (unstimulated) saliva, and the parotid glands are the major contributors to stimulated saliva. The contribution of sublingual glands to unstimulated and stimulated whole saliva is low.

METHODS OF COLLECTING SALIVA[53]

Several methods for collecting saliva have been reported and tested for validity and reproducibility. There are different methods for collection of whole saliva and gland specific saliva. Salivary constituents vary depending on the harvesting method and the degree of salivary flow. Irrespective of the method used, subjects should be instructed to clean the oral cavity before collection by rinsing the mouth thoroughly with water to avoid contaminants.

STIMULATED SALIVA

Stimulated saliva is usually collected by inducing masticatory action on paraffin wax or chewing gum (i.e. absorbent method) or gustatory stimulation by application of citric acid on the subject's tongue to increase the salivary flow rate. This method obviously affects the quantity and pH of the saliva, and is generally only used in patients who have difficulty in producing enough saliva[53]

UNSTIMULATED SALIVA

Unstimulated saliva is collected without exogenous facilitation, and its flow rate is mostly affected by the degree of hydration, olfactory stimulation, exposure to light, seasonal and diurnal factors. The three most common approaches for collection of unstimulated saliva are draining, spitting, and suction methods[43]

METHODS FOR COLLECTION OF WHOLE SALIVA

PASSIVE DROOL

A convenient method often used is the collection of whole saliva by passive drool into a small vial. Passive drool is highly recommended because it is approved for use with almost all analytes, unlike absorbent devices, which can sometimes cause interference in immunoassays. It is important to use high-quality polypropylene vials, since other vials can lead to problems with analyte retention or the introduction of contaminants that can interfere with the immunoassay. The vials use must also seal tightly and be able to withstand temperatures as low as -80ºC[37]

ORAL SWAB

Oral Swab is an excellent alternative to passive drool because of its ease of use. The oral swab is made of a non-toxic, inert polymer shaped into a 30 x 10 mm cylinder. It is not recommended for children under the age of six due to the possibility of a choking hazard[54]

COLLECTION OF GLAND SPECIFIC SALIVA

Saliva from individual glands is not contaminated with food debris and microorganisms, so it is prudent to acquire saliva from individual major glands. The techniques are tedious and require custom-made collection devices.

PAROTID GLAND: The parotid gland secretion is voided in the oral cavity via the Stensen duct at the vicinity of the parotid papilla opposite the maxillary second molar. A modified Lashley cup or Carlson-Crittenden collector often is used for collecting saliva from the parotid glands.[53]

SUBMANDIBULAR AND SUBLINGUAL GLANDS: The submandibular and sublingual gland secretions are voided in the oral cavity via the Wharton duct, which opens into the floor of the mouth. Many custom-made collectors such as the Wolff collector are used

MINOR SALIVARY GLANDS: Minor salivary gland secretions do not have much clinical application, owing to the labor-intensive nature of collecting them. Specific methods like palatal acrylic devices are used to measure minor salivary gland secretions.[37]

TECHNIQUES FOR ANALYSIS OF SALIVA

Salivary diagnostics is a dynamic and emerging field in the diagnosis of oral and systemic diseases. Saliva is highly informative and has a potential for new approaches to prognosis, laboratory or clinical diagnosis, and monitoring and management of patients with both oral and systemic diseases. Many different techniques have been used for the past two decades for the analysis of saliva. Out of these techniques proteomics revealed disease specific biomarkers for various pathologic conditions. This would eventually lead to the invention and establishment .

PROTEOMICS

'Proteomics is a multifaceted approach to study various aspects of protein expression, post-translational modification, interactions, organization and function at a global level[43] . Saliva harbours a dynamic proteome with a wide range of proteins. The analysis of salivary proteome is indeed a challenging procedure as it contains a large number of proteins within an extremely wide concentration range. Different proteomic technologies have been used for detection and quantification of the salivary proteome. Based on advanced mass spectrometry different protein separation technologies have been used specifically or in combinations. The process of initial biomarker fractionation is required for mass spectrometry based proteomic techniques. Each fraction or spot of a protein is subjected to certain mass-spectrometric technology. The mass data obtained as a result is then used to search protein, geneomic expressed sequence tag (EST), and other species-specific databases to identify proteins present in each selected spot[55]. By comparing different samples, the changes in level of

expression of individual proteins can be identified and quantified. This permits the identification of biomarkers associated with specific pathologic or physiologic states.

There are four different techniques used individually or in combination in biomarker screening and identification. These include matrix-assisted laser desorption ionization – time of flight/mass spectrometry (MALDI-TOF/MS), 2DE, 2D-liquid chromatography /mass spectrometry (LC/MS), and surface enhanced laser desorption/ ionization time-of-flight mass spectrometry (SELDI-TOF/MS).[56]

In a study Shotgun proteomics based on reversed phase liquid chromatography (LC) off-line prefractionation of intact proteins and subsequent LC-tandem mass spectrometry (LC-MS/MS) analysis of proteolyzed peptides was used for profiling of saliva proteins from OSCC or matched control subjects. Identification of the peptides and represented proteins was done by using the Mascot database search engine. 52proteins were found to be present in OSCC but are absent in the healthy control subjects whereas 29 proteins were found only in the healthy subjects but absent in OSCC patients. Five candidate biomarkers M2BP, MRP14, CD59, profilin 1, and catalase were successfully validated using immunoassays on an independent set of OSCC patients and matched healthy subjects.[57]

Analysis of the pooled soluble fraction of whole saliva from four subjects with pre-malignant lesions and four with malignant lesions by mass spectrometry and prioritization of candidate biomarkers via bioinformatics with validation of selected proteins by western blotting revealed increased levels of myosin and actin in patients with malignant lesions.[58]

Identification of candidate protein and mRNA biomarkers of primary Sjogren syndrome in WS samples by Mass spectrometry and expression microarray profiling revealed sixteen WS proteins down-regulated and 25 WS proteins up-regulated in primary Sjogren Syndrome patients compared with matched healthy control subjects.[59]

Multidimensional liquid chromatography/tandem mass spectrometry (2D-LC-MS/MS) with Label-free quantification has been used to identify protein biomarkers in whole saliva from type-2 diabetic individuals and compared with selected protein biomarkers were then validated in saliva from control, diabetic, and prediabetic subjects by Western immunoblotting and ELISA. This identified a total of 487 unique proteins. Approximately 33% of these proteins have not been previously reported in human saliva. Of these, 65 demonstrated a greater than 2-fold difference in levels between control and type-2 diabetes samples.[60]

MICROARRAY

Microarray-based assay technology provides investigators with the ability to measure the expression profile of thousands of genes in a single experiment. With the use of a QIAamp Viral RNA kit RNA was isolated from saliva supernatant and aliquots of RNA were treated with RNase-free DNase and then subjected to linear amplification in a RiboAmp™ RNA Amplification kit. Gene expression analysis of these samples using the Human Genome U133A Array. Two hundred seven probe sets, representing 185 genes, were detected among all 10 participants. 570 genes present in 8/10 (80%) arrays, and 417 genes in 9/10 (90%) arrays were found using the same criterion. Among these, 49 and 37 salivary mRNAs, respectively, were found to be present in saliva at protein levels as determined by proteomic approaches. The

genes present among all 10 arrays (100%) were referred to as "Normal Saliva Core Transcriptome (NSCT).[61]

POLYMERASE CHAIN RECTION (PCR) AND IMMUNOASSAYS

The techniques like quantitative PCR and ELISA are used initially to validate the biomarkers identified as a result of proteomic and transcriptomic technologies. In a study using reverse transcriptase preamplification- quantitative PCR (RT-preamp-qPCR) in 12 healthy controls a total of 314 miRNAs were measured. Selected miRNAs were validated in saliva of 50 oral squamous cell carcinoma patients and 50 healthy matched control subjects. Significantly lower levels of miR-125a and miR-200a were detected in the saliva of OSCC patients than in control samples.[62].

Assessment of salivary profiles of patients with Erosive lichen planus by ELISA after being treated with prednisolone revealed a significantly higher level of IFN-γ, TNF-α, and sTNFR-2 was detected in patients before treatment than in controls. The salivary levels of IFN-γ, TNF-α and sTNFR-2 were found to be decreased significantly following treatment, when compared to their pre-treatment levels.[47]

Detection of levels of proinflammatory cytokines Interleukin 1α (IL-1 α), Interleukin (IL-6), Interleukin (IL-8) and Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) was done by enzyme linked immunosorbent assay (ELISA). Higher concentrations of Serum IL-6 and IL-8 level were detected in patients with OSCC than the control group. And the levels of IL-1 alpha, IL-6, IL-8 and GM-CSF in saliva showed significant increase in patients with OSCC when compared with control group. Serum and salivary IL-8 were proved to be useful in the diagnosis of OSCC patients and separating between OSCC patients and control group.[63]

Simple and inexpensive sampling method is a compelling reason to make saliva a reliable diagnostic tool. Moreover it causes minimal subject discomfort and is a source of definitive disease-associated protein and genetic markers. The advantage of easy collection, storage, shipping, and voluminous sampling, makes saliva a better diagnostic biofluid than serum or urine. It is also easier to handle saliva during diagnostic procedures than blood. Thus saliva can be used as an accurate, portable and easy-to-use diagnostic platform.

LIMITATIONS OF USING SALIVA AS A DIAGNOATIC TOOL

Although saliva has many advantages in case of ease of sample collection and handling it also carries some limitations. The level of certain markers in saliva is not the exact amount present in serum. A defined relationship is required between the concentration of the biomarker in serum and the concentration in saliva for a proper diagnosis.

The detection of salivary biomarkers with diagnostic value depends on the normal functioning of the salivary glands. The degree of stimulation of salivary flow and the method of collection highly influences the composition of saliva. Concentration and availability of the biomarkers are affected by the changes in salivary flow rate and pH. Salivary flow rate varies between individuals and in the same individual under various conditions (diurnal variations, degree of hydration).

Many serum markers and proteins can enter saliva through gingival crevicular fluid (GCF). This may affect the diagnostic quality of many salivary markers. Moreover certain systemic disorders, medications, and radiation may affect salivary gland function and consequently the quantity and composition of saliva.

In the technical aspect the most serious disadvantage of saliva is the instability if its constituents. This is mainly due to the proteolytic enzymes derived from the host and from oral micro-organisms. These enzymes can significantly affect the stability of certain diagnostic markers. Some molecules are also degraded during intracellular diffusion into saliva. Advent of many chemicals like protease inhibitors, ethanol etc and storage of the samples under very low temperature ($^{-}80^{o}$celsius) has helped the researchers to overcome this limitation to an extent.[42,64,65]

RECENT ADVANCES AND USES

The first step in the growth of saliva as a diagnostic tool is the discovery of informative biomarkers. Biomarker discovery in saliva is done by molecular techniques like genomics,proteomics, transcriptomics and metabolomics as mentioned before. With these techniques the genes, proteins, mRNAs and metabolites in saliva have been characterized by Based on these platforms different advanced technologies like microfluidics and micro/nano electromechanical system (MEMS/NEMS) have been invented to serve as point of care devices. In this system multiple analytes in a drop of saliva could be simultaneously measured and analyzed through highly sensitive biosensors.[65]

The device invented in UCLA, California has been labelled the Oral Fluid NanoSensor Test (OFNASET). The OFNASET is a point-of-care, automated, and easy-to-use integrated system that will enable simultaneous and precise detection of multiple salivary proteins and nucleic acids. In addition, this system is portable and could be used not only in the doctor's office, but also in any other healthcare station to perform an instant point-of-care diagnosis[43]

INTERLEUKIN 1 B (IL1B)

IL-1 was first described as a protein that induced fever and was called human leukocytic pyrogen and also it was known previously as lymphocyte activating factor (LAF). IL 1 is a 17-kilodalton (kD) glycoprotein which is made up of 2 major proteins, IL-1A and IL-1B.[66] There are 11 members of the IL-1 family. IL-1A and IL-1B have minimal sequence homology but they have similar biological properties. They differ from each other fundamentally in their localization, maturation, and secretion. IL-1 is a pluripotent cytokine responsible for normal physiological roles including induction of vascular permeability, fever, secretion of additional cytokines in autoimmune diseases, production and release of prostaglandins, pituitary hormones, and collagenases and stimulation of the immune system to produce lymphocytes. An important balance exists between the beneficial and harmful effects of IL-1. IL-1 acts as an endogenous pyrogen and is a potent proinflammatory cytokine. It has potentiating effects on cell proliferation, differentiation, and function of many innate and specific immunocompetent cells. IL-1 initiates and potentiates immune and inflammatory responses and thereby mediates many inflammatory diseases. The IL-1 receptor antagonist (IL-1Ra) is synthesized and released in response to the same stimuli that lead to IL-1 production.[67]

In human breast carcinoma IL-1 levels were significantly higher in invasive carcinoma than in ductal carcinoma in situ or in benign lesions, implying that elevated levels of IL-1 are directly correlated with a more advanced disease.[68] Neoplastic cells directly produce IL-1 or can induce cells within the tumor microenvironment to do so. IL-1 shows various expression patterns: it is expressed in an autocrine or paracrine fashion. It stimulates the tumor cell by exhibiting autocrine behaviour by itself to

invade and proliferate and it can also exert paracrine effects on stromal cells in the tumor microenvironment. The exact mechanisms by which IL-1 promotes tumor growth remain unclear, though the protein is believed to act primarily indirectly (Figure 3). IL-1 induces expression of matrix metallo proteinases (MMP) and stimulates nearby cells to produce angiogenic proteins and growth factors such as VEGF, IL-8, IL-6, TNFα, and tumor growth factor beta (TGFβ) which helps in invasion and metastasis.(Annexure VIII) In the tumor microenvironment IL-1 has local effects on host infiltrating cells that result in production of proangiogenic and prometastatic mediators. It has been shown in some studies that IL-1 is necessary for tumor growth, metastasis, and angiogenesis.[18,19,69]

In a study where the amounts of IL-1 beta in the biopsied specimens of normal oral mucosa, oral submucous fibrosis (OSF), oral squamous cell carcinoma (OSCC), and verrucous hyperplasia (VerH) were quantified by ELISA, a significant difference in IL-1 beta level was found between normal oral mucosa and OSCC . Immunohistochemistry staining technique using antibody against IL-1 beta showed positive staining in hyperplastic epithelium and neoplastic cells of OSCC. The amount of IL-1 beta did not change significantly as OSF progressed. The gradual increase in the levels of IL-1 beta from normal oral mucosa through verrucous hyperplasia to oral squamous cell carcinoma suggests that IL-1 beta plays an important role in oral carcinogenesis.[70]

Distinct mRNA expression patterns can be identified in saliva from cancer patients, and the differentially expressed transcripts has been studied which can serve as biomarkers for cancer detection. In a study where Microarray analysis of unstimulated saliva collected from 32 patients with primary T1/T2 OSCC and 32

normal subjects with matched age, gender, and smoking history showed there are 1,679 genes exhibited significantly different expression level in saliva between cancer patients and controls. Seven cancer-related mRNA biomarkers that exhibited at least a 3.5-fold elevation in OSCC saliva were consistently validated by qPCR on saliva samples from OSCC patients and controls. These salivary RNA biomarkers are transcripts of IL8, IL1B, DUSP1, HA3, OAZ1, S100P, and SAT of which IL1B showed 5.48 fold increase in OSCC when compared to normal controlsThe combinations of these biomarkers yielded sensitivity (91%) and specificity (91%) in distinguishing OSCC from the controls. The utility of salivary transcriptome diagnostics is successfully demonstrated in this study for oral cancer detection.[71]

To know if the above mentioned biomarkers are (IL8, IL1B, DUSP1, HA3, OAZ1, S100P, and SAT) discriminatory in cohorts of different ethnic background (Serbian population), mRNA transcripts of DUSP1, IL8, IL1B, OAZ1, SAT1, and S100P and three proteins IL1B, IL8, and M2BP were tested on 18 early and 17 late stage OSCC patients and 51 healthy controls with quantitative PCR and ELISA. Four transcriptome (IL8, IL1B, SAT1 and S100P) and all proteome biomarkers were significantly elevated in OSCC patients when compared to controls. The sensitivity/specificity for OSCC total was 0.89/0.78, for T1–T2 0.67/0.96, and for T3–T4 0.82/0.84. Seven of the nine salivary biomarkers (three proteins and four mRNAs) showed highly significant performance in late stage cancer. IL1B showed 3.96 fold increase in OSCC when compared with normal controls. The previously discovered and validated salivary OSCC biomarkers were proved to be discriminatory and reproducible in a different ethnic cohort.[9]

In a study using Luminex Multianalyte Profiling (xMAP) technology for measurement of salivary proteins for IL1B and IL8 in 20 OSCC patiens and controls, both the markers were significantly elevated[72]And the measured levels of IL-8 in the saliva of OSCC subjects from this study were also comparable with those measured by ELISA from another group of researchers (Rhodus *et al*, 2005) [73]

INTERLEUKIN 8 (IL8)

Interleukin-8, also known by different other names: CXCL 8, monocyte-derived neutrophil chemotactic factor (MDNCF) and neutrophil attractant/ activation protein (NAP- 1) is a 10 kD glycoprotein and was originally isolated from human peripheral blood monocytes.[57] There are two cell-surface G protein–coupled receptors for IL 8 termed as CXCR1 and CXCR2 which mediate it's the biological effects. Expression of IL-8 is primarily regulated by activator protein and/or nuclear factor-kappa B (NF-κB) mediated transcriptional activity. Expression of IL-8 has been shown to be regulated by a number of different stimuli including inflammatory signals (e.g., tumor necrosis factor a, IL-1h), chemical and environmental stresses (e.g., exposure to chemotherapy agents and hypoxia), and steroid hormones (e.g., androgens, estrogens, and dexamethasone).[19]

Increased expression of IL-8 and/or its receptors has been characterized in cancer cells, endothelial cells, infiltrating neutrophils, and tumour-associated macrophages. This suggests that IL-8 may function as a significant regulatory factor in the tumour microenvironment. The induction of IL-8 signalling activates multiple upstream signalling pathways (Annexure IX). These pathways regulate numerous transcription factor activities which result in gene expression. This also modulates the

cellular proteome at the level of translation and also effect the organization of the cell cytoskeleton through post translational regulation of regulatory proteins. IL-8 signalling promotes angiogenic responses in endothelial cells, increases proliferation and survival of endothelial and cancer cells, and potentiates the migration of cancer cells, endothelial cells, and infiltrating neutrophils at the tumour site as a result of the diversity of effectors and downstream targets.

Secretion of IL-8 from cancer cells enhances the proliferation and survival of cancer cells through autocrine signaling pathways. Tumor derived IL-8 can activate endothelial cells in the tumor vasculature to promote angiogenesis and induce a chemotactic infiltration of neutrophils into the tumor site. IL-8 also contributes to promote cell invasion and migration. The induction of tumor-associated macrophages to secrete additional growth factors by IL-8 will further increase the rate of cell proliferation and cancer cell invasion at the tumor site. The ability of IL-8 signalling to induce different cell types present within the tumor microenvironment suggests that targeting of CXC-chemokine may have implications on limiting disease progression and it also have potential to assist in sensitizing tumors to chemotherapeutic and biological agents.[68] IL-8 is found as a useful serum marker for predicting the prognosis of pancreatic cancer.

In a previously mentioned study where distinct mRNA expression patterns of IL8, IL1B, DUSP1, HA3, OAZ1, S100P and SAT were identified in saliva from cancer patients with microarray and consistently validated by qPCR on saliva samples from OSCC patients and controls, IL8 performed the best among others giving 24.3 fold increase, 88% sensitivity and 81% specificity ensuring the utility of IL8 in oral cancer detection.[71]

In another study which was done to evaluate these markers in a different cohort with quantitative PCR and ELISA, among the other transcriptome and proteome biomarkers IL8 showed 3.9 fold increase and proved to be discriminatory and reproducible in a different ethnic cohort.[9]

IL 6 and IL 8 has been investigated in mRNA and protein levels in saliva and serum of patients with OSCC and controls using quantitative polymerase chain reaction PCR and ELISA respectively. IL-8 at both the mRNA and protein levels was detected in higher concentrations in the saliva of patients with OSCC compared with controls. Significant difference in the amount of IL-8 mRNA expression has been identified. The level of IL-8 in the saliva of OSCC patients was 720 pg/mL which was significantly higher than that in the saliva of the control group which was 250 pg/mL. A comparison of the total protein concentrations in saliva between the 2 groups was done in order to ensure that the elevated levels of IL-8 protein in saliva were not due to an elevation of total protein levels in the saliva of patients and no significant differences were found. There was no significant difference in the IL-6 levels between the 2 groups in the salivary concentration at either the mRNA or the protein level. In contrast the serum analysis showed that levels of IL 6 was significantly elevated in OSCC patients with insignificant IL 8 levels.[11]

The levels of proinflammatory cytokines IL-1, IL-6, IL-8 and Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) have been investigated by ELISA. IL-6 and IL-8 levels in serum were detected at higher concentrations in patients with OSCC than the control group. No significant differences in serum IL-1 alpha and GM-CSF of patients with OSCC was identified when compared with control group. The levels of IL-6, IL-8, IL-1 alpha and GM-CSF showed significant increase in saliva of

patients with OSCC when compared with control group. Serum IL-6 was more significant in OSCC patients than salivary IL-6. Both serum and salivary IL-8 were significant in OSCC patients and it is proved to be useful in separating OSCC patients and control group.[62]

In another study where the concentration of tumor necrosis factor α, interleukin 1α, 6, and 8 in the saliva of oral squamous cell carcinoma patients with control group were compared using ELISA, the concentration of salivary interleukin 6 in oral squamous cell carcinoma patients was higher than control group and it was statistically significant. The concentration of salivary tumor necrosis factor α, interleukin 1α and 8 in case group was higher than control group but it was not statistically significant.[74]

Higher levels of IL 1B, IL 8, IL6 and osteopontin have been identified in OSCC when compared to controls using ELISA. But the difference was shown to be significant only in IL 6 concentrations.[75] Estimation of serum and salivary samples of patients with OSCC and OPMD for a panel of tumor markers Cyfra 121-1, IL 6, IL 8, and TNF alpha by ELISA revealed significant elevation of levels of Cyfra 121-1, IL 6, IL 8, and TNF alpha in IL 6, IL 8 in saliva. The levels of these markers were significantly higher in serum also except for Cyfra 121-1.[46]

Another study on proangiogenic and proinflammatory cytokines in 2005 which was done on TNF-alpha, IL-1, IL-6, and IL-8 showed elevated levels of IL 8 in the saliva of patients with OSSC and OPML (oral potentially malignant lesions) as compared to controls.[7 3] Result of a study in India done on 25 OSCC patient by ELISA suggested that salivary IL-8 can be utilised as a potential biomarker for OSCC but it was found to be non-conclusive for oral pre-malignancy.[76]

Results

The study group comprised of 72 subjects including 18 subjects with OSCC, 30 with OPMD and 24 controls grouped under Group I, Group II and Group III respectively. Group II was subdivided into subjects with oral submucous fibrosis (Group II A) and subjects with leukoplakia (Group II B). Group III comprised of controls including 12 patients with periodontitis and 12 healthy controls. (Graph 1, Table 1) As the sample size was small OSCC and leukoplakia was not further stratified based on differentiation and level of dysplasia respectively.

Gender and age distribution among study groups

Among the overall study population (n=72) 80.6% were males (n=58) and 19.4% were females (n=14). In Group I (n=18) 88.9% (n=16) were males and 11.1% (n=2) were females. In Group II A all the subjects (100%) were males (n=16). In Group II B (n=14) 92.9% (n=13) were males and this group included only one female (7.1%). In Group III (n=24) 54.2% (n=13) were males and 45.8% (n=11) were females. (Graph 2, Table 2)

The age of the subjects ranged from 25 to 83 years with the mean of 42.1 ± 12.5. The age distribution among Groups I, II A, II B and III are 51 ± 14.9, 39.3 ± 10, 42.4 ± 12.6 and 37.1 ± 8.5 respectively. (Graph 3, Table 3)

Prevalence of habits among study groups

The number of subjects who had the habit of smoking were 11 (61.1%), 6 (37.5%), 6 (42.9%), and 3(12.5%) in Groups I, II A, II B, and III respectively. Group I included 16 (88.9) subjects while all subjects in Group II A (100%) and 10 (71.4%) subjects in Group II B had chewing habit. The number of subjects who had drinking

habit was 8 (44.4%), 13 (81.3%) and 10 (71.4%) in Group I, II A and II B respectively.(Graph 4, Table 4) No subjects in Group III had chewing or drinking habit.

Levels of Interleukins in study groups

IL 8

The mean concentration of IL 8 in the study population was found to be 1079.31 ± 424.57 in Group I, 629.95 ± 590.01 in Group II A, 711.5 ± 366.25 in Group II B and 671.53 ± 485.17 in Group III.(Graph 5, Table 5) The differences in the groups were statistically significant (P=0.023). Multiple comparisons between the groups was done using Tukey HSD test.

The concentration of IL 8 was found to be elevated in Group I when compared to other groups. Comparison of Group I with group II A (P=0.038) and Group III (P=0.038) was statistically significant while the comparison with Group II B showed no statistical significance (P=0.143). Comparison of Group II A with Group II B (P=0.966) and Group III (P=0.993) also showed no statistical significance. Group II B and Group III also showed no statistical significance (P=0.995) when compared.

IL 1B

The mean concentration of IL 1 B was found to be 1086.14 ± 758.29, 514.25 ± 505.7, 1073.84 ± 799.93 and 535.36 ± 477.88 in Groups I, IIA, IIB and III respectively. (Graph 6, Table 6) The differences in the groups were statistically significant (P=0.006). In multiple comparisons, comparison between Group I and Group III only was statistically significant (P=0.033). Comparisons between other

groups Group I with Group II A (P=0.05) and Group II B (P=1.0), Group II A with Group II B (0.83) and Group III (1.0) and Group II B with Group III (P=0.64) were statistically not significant.

Comparison with group IIIA and B separately

When IL 8 and IL1B concentrations were compared with the control group with periodontitis (group IIIA) and without periodontitis (group IIIB), we found group IIIA and B showed no statistical significance when compared separately with groups I, IIA and IIB.(Graphs 7 and 8, tables 7 and 8).

Tables and Graphs

Graph 1

Distribution of number of samples in different groups

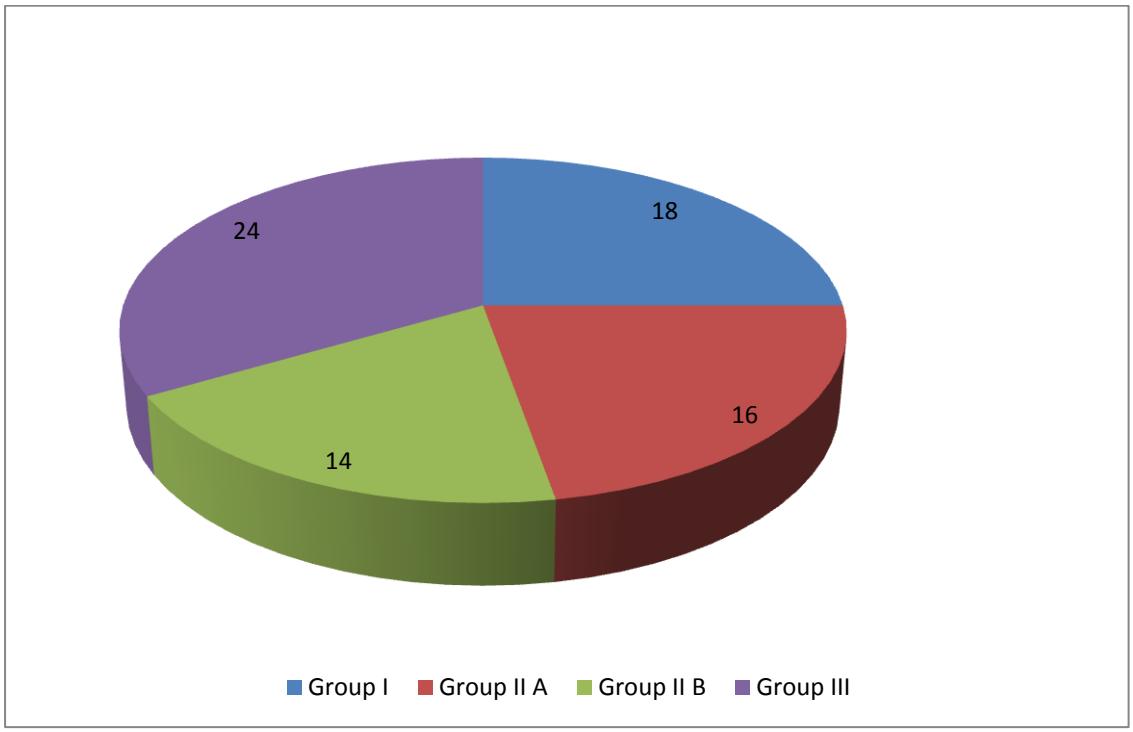


Table 1

Distribution of number of samples in different groups

Groups	Type of sample	Sample size
Group I	Oral squamous cell carcinoma	18
Group II A	Oral submucous fibrosis	16
Group II B	Leukoplakia	14
Group III(A and B)	Controls (Normals)	24

Graph 2

Distribution of gender in study groups

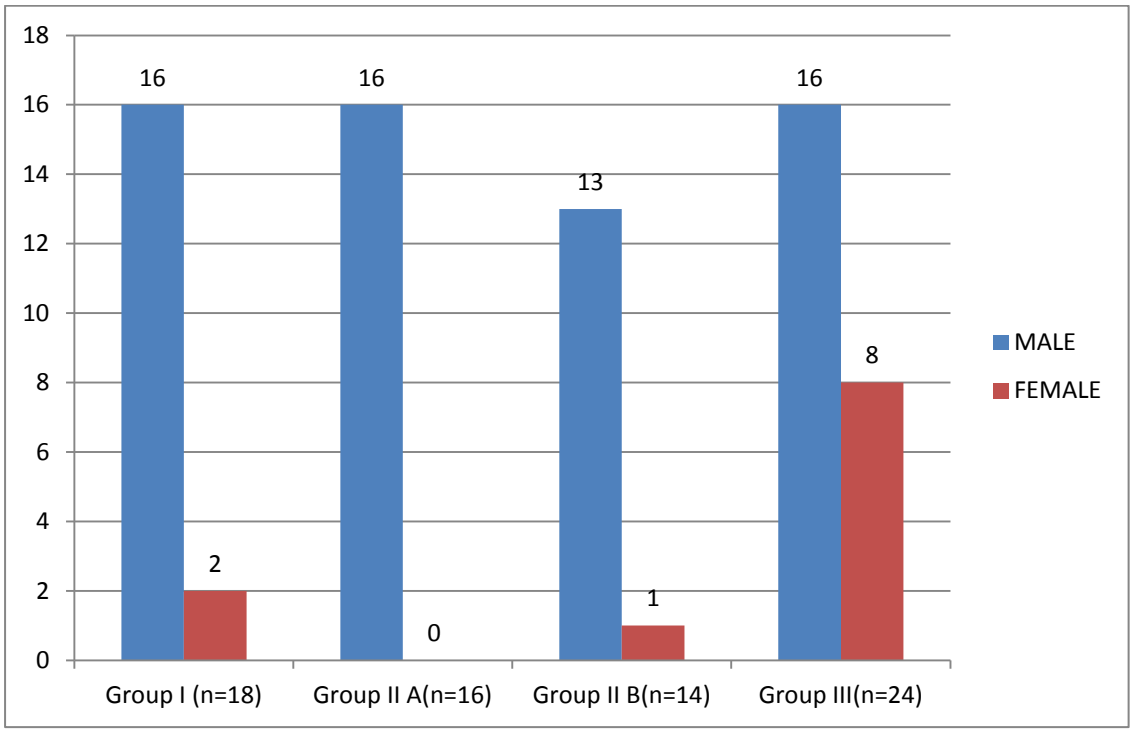


Table 2

Distribution of gender in study groups

Study Groups	**Male**		**Female**	
	n	Percentage	n	Percentage
Group I(n=18)	n=16,	88.9%	n=2	17.9%
Group II A(n=16)	n=16	100%	n=0	0%
Group II B(n=14)	n=13	92.9%	n=1	7.1%
Group III(n=24)	n=13	54.2%	n=11	45.8%

Graph 3

Distribution of age in study groups

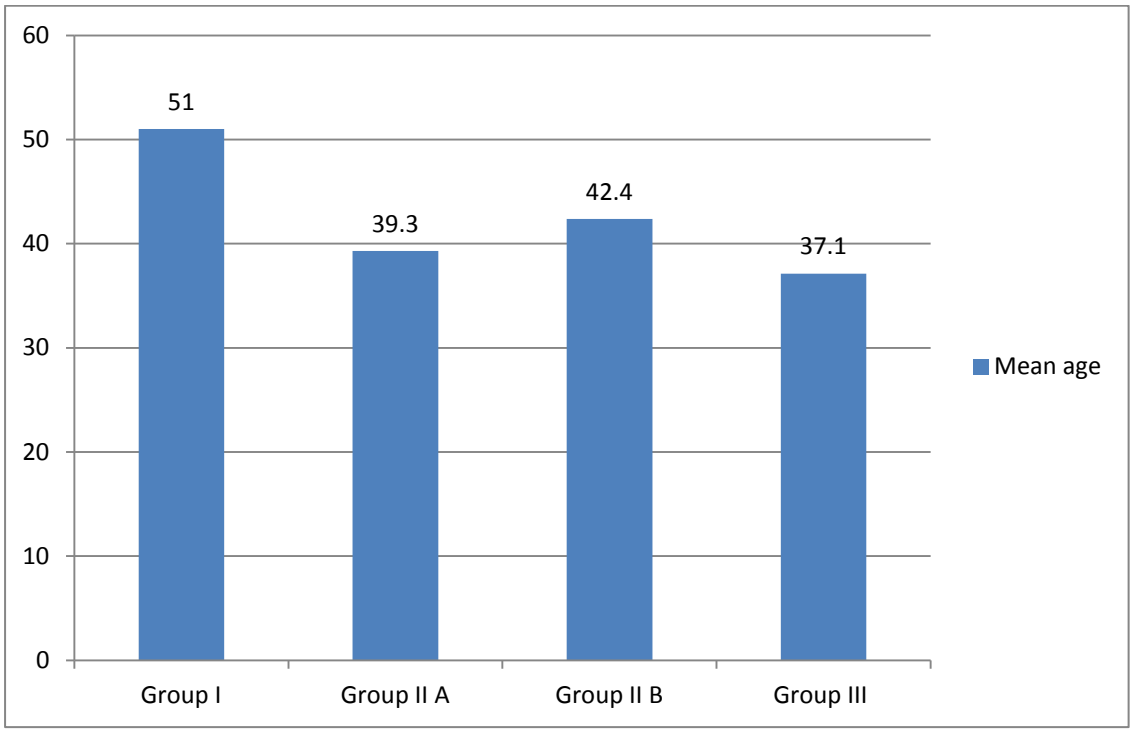


Table 3

Distribution of age in study groups

Study Groups	Mean age	Standard deviation
Group I(n=18)	51	14.7
Group II A(n=16)	39.3	9
Group II B(n=14)	42.4	12.6
Group III(n=24)	37.1	8.4

Graph 4

Prevalence of habits in the study groups

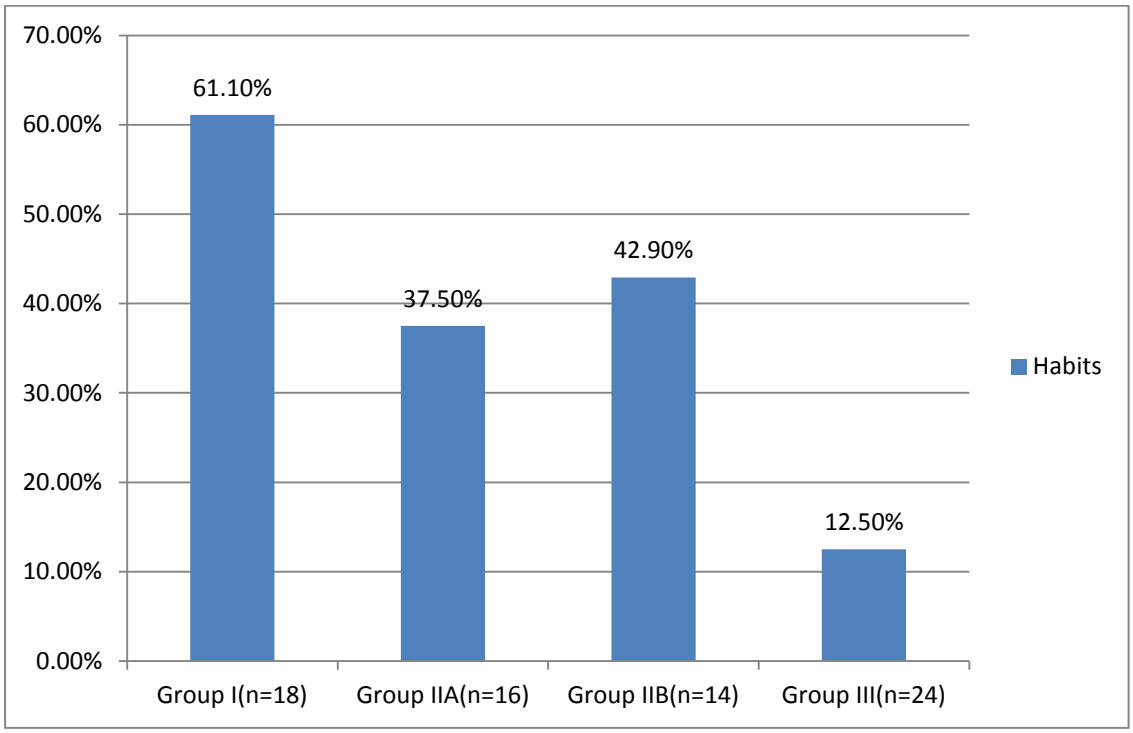


Table 4

Prevalence of habits in the study groups

Study Groups	**Smoking**		**Chewing**		**Alcohol**	
	n	Percentage	n	Percentage	n	Percentage
Group I(n=18)	11	61.1%	16	88.9%	8	44.4%
Group II (n=16)	6	37.5%	16	100%	13	81.3%
Group II B(n=14)	6	42.9%	10	71.5%	10	71.4%
Group III(n=24)	3	12.5%	0	0%	0	0%

Graph 5

IL 8 concentrations in study groups

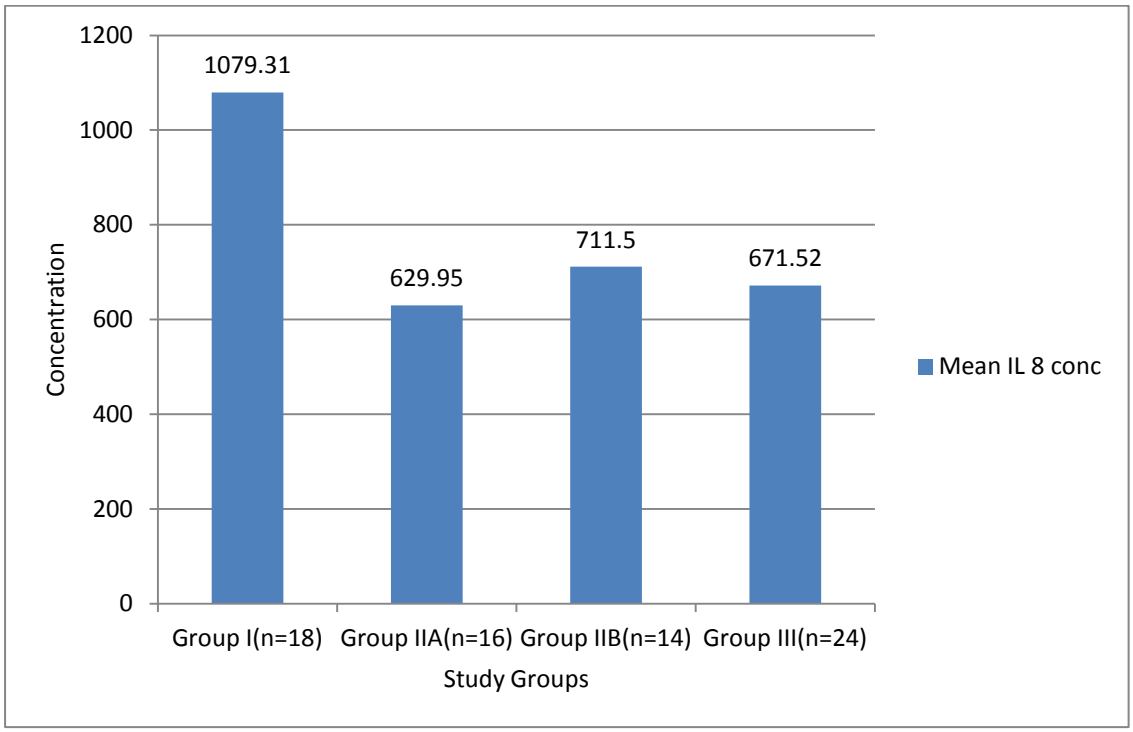


Table 5

IL 8 concentrations in study groups

Study Groups	Mean	Standard deviation	P value
Group I(n=18)	1079.31	424.57	0.023*
Group II A(n=16)	629.95	590.01	
Group II B(n=14)	711.5	366.25	
Group III(n=24)	671.52	485.17	

*Statistically significant at 5% level, P <0.05

Graph 6

IL 1B concentrations in study groups

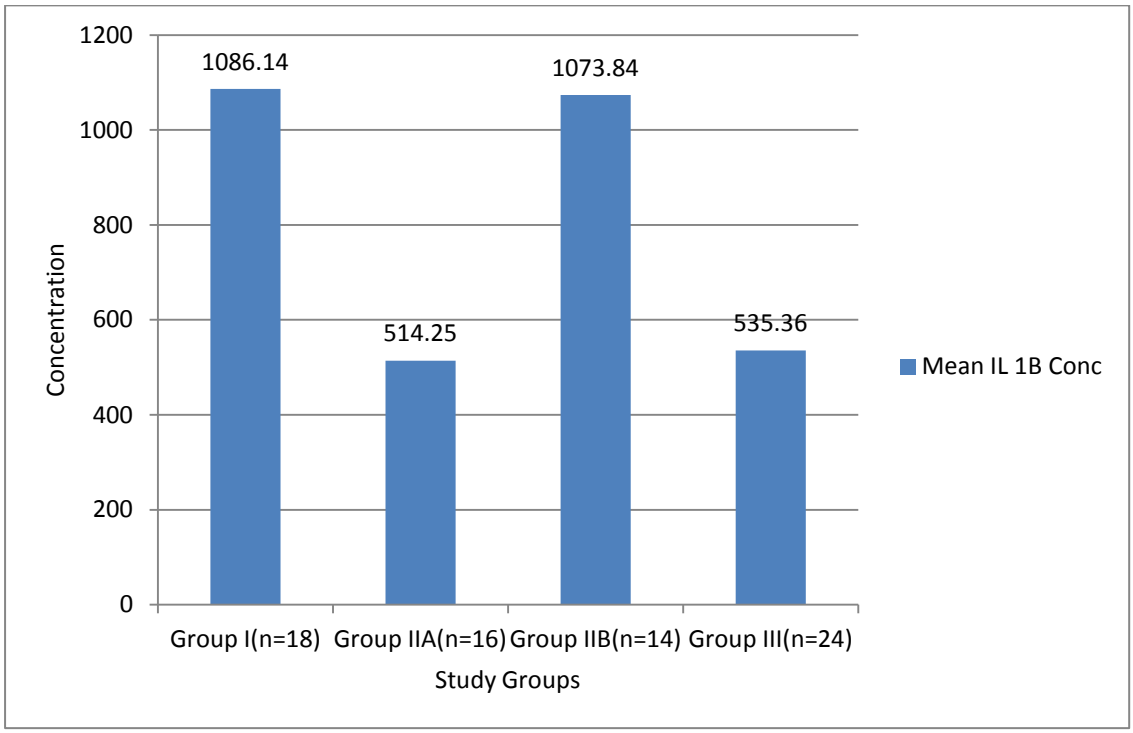


Table 6

IL 1B concentrations in study groups

Study Groups	Mean	Standard deviation	P value
Group I(n=18)	1086.14	758.29	0.006*
Group II A(n=16)	514.25	505.79	
Group II B(n=14)	1073.84	799.93	
Group III(n=24)	535.36	477.88	

*Statistically significant at 5% level, P <0.05

Graph 7

Comparison of mean differences in IL 8 concentration between group I, IIA and IIB with group IIIA and IIIB

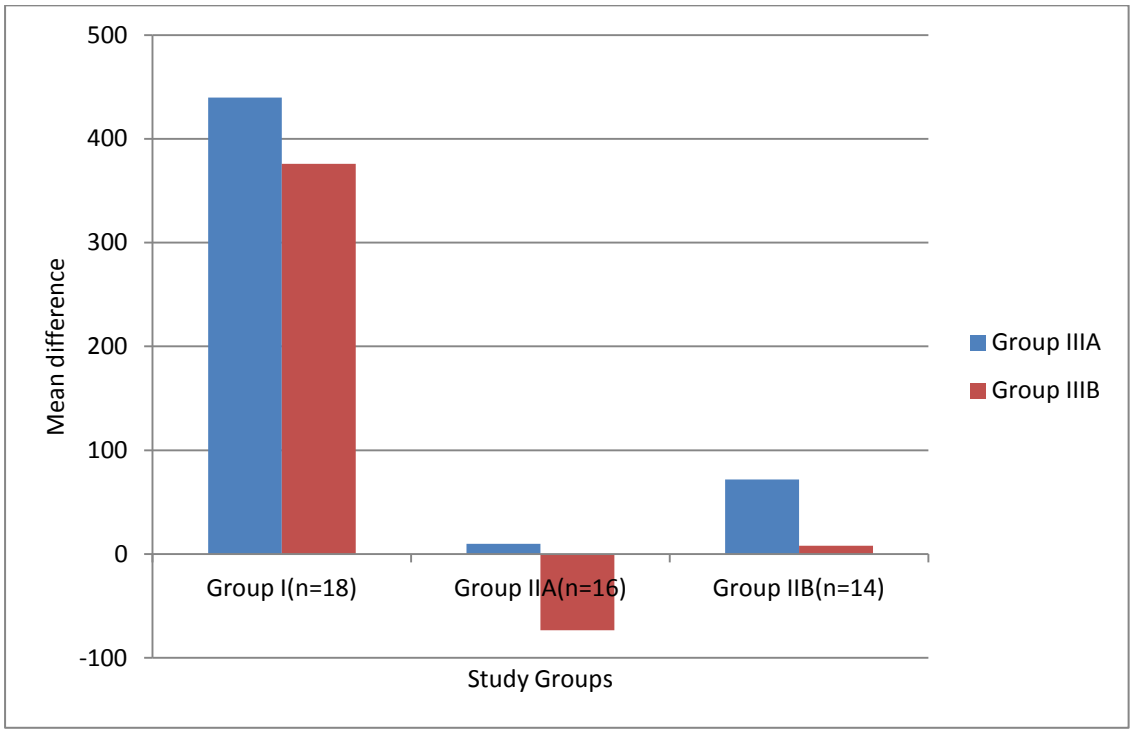


Table 7

Comparison of mean differences in IL 8 concentration between group I, IIA and IIB with group IIIA and IIIB

Groups	**Mean difference with Group IIIA**	**P value**	**Mean difference with Group IIIB**	**P value**
Group I(n=18)	439.58	0.1	375.99	0.23
Group IIA(n=16)	9.78	1.0	-73.36	0.99
Group IIB(n=14)	71.76	0.99	8.18	1.0

Graph 8

Comparison of mean differences in IL 1B concentration between group I, IIA and IIB with group IIIA and IIIB

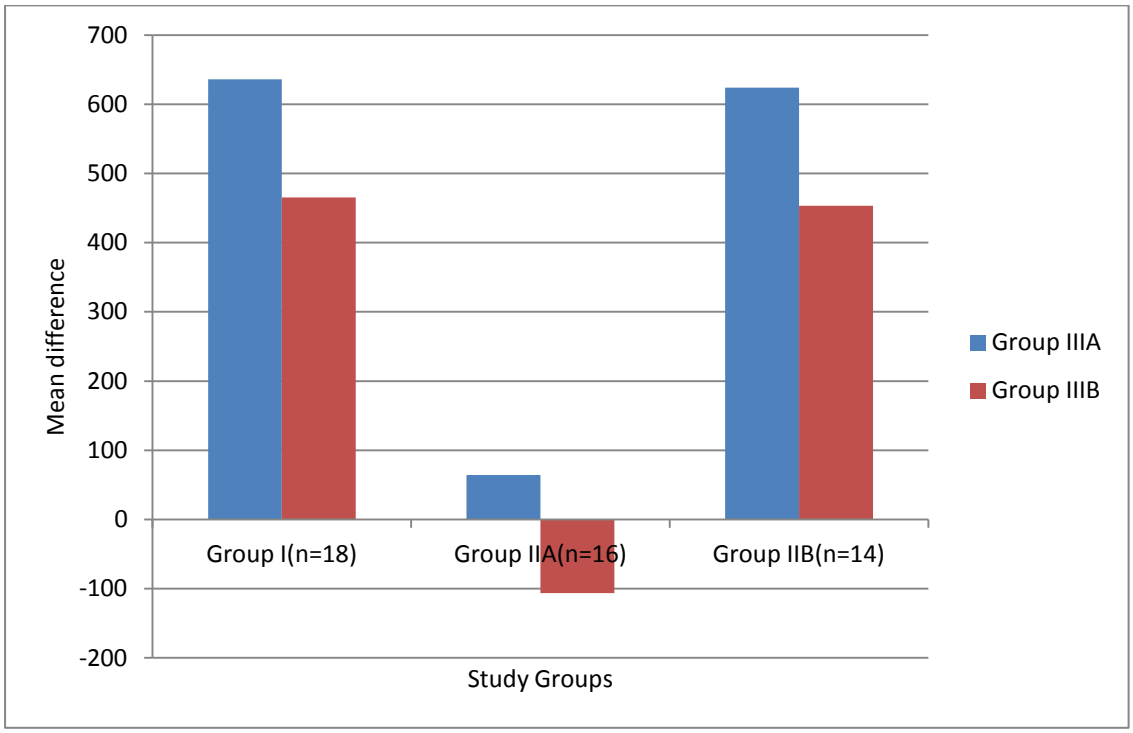


Table 8

Comparison of mean differences in IL 1B concentration between group I, IIA and IIB with group IIIA and IIIB

Groups	**Mean difference with Group IIIA**	**P value**	**Mean difference with Group IIIB**	**P value**
Group I(n=18)	636.07	0.06	465.5	0.29
Group IIA(n=16)	64.18	0.99	-106.4	0.99
Group IIB(n=14)	623.77	0.1	453.19	0.37

Figures

ARMAMENTARIUM

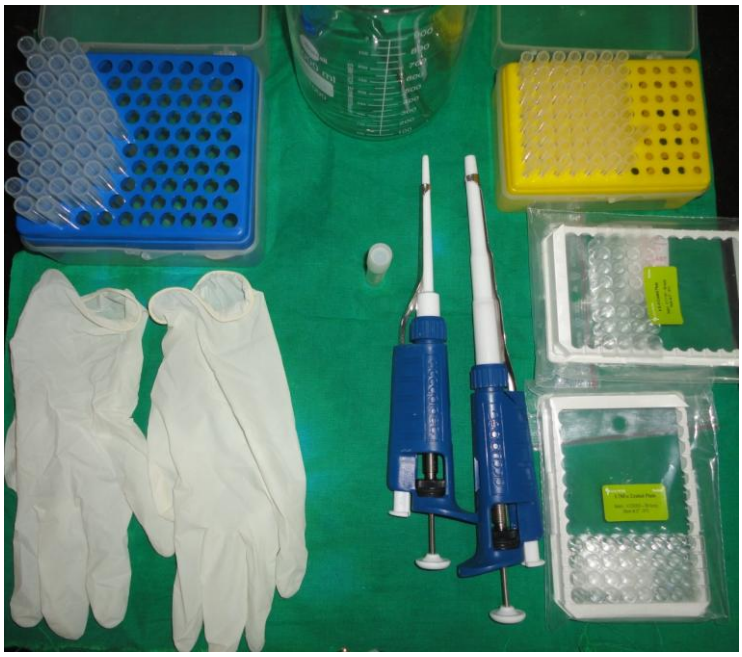


Figure 1: Armamentarium Used For Specimen Handling and Elisa Procedure

Figure 2: Cooling Centrifuge

Figure 3: Micro-Centrifuge Tube

Figure 4: IL 8 ELISA kit with reagents

Figure 5: IL IB ELISA kit with reagents

Figure 6: ELISA plate reader and washer

Discussion

Oral cancer is a serious debilitating disease which is a major health problem in our country[3,6]. As OSCC is associated with habits and occur mostly as a result of progression from precancerous states, early detection or prevention of OSCC is not too hard to achieve if there is an easier and non-invasive technique than biopsy for screening of people at risk. The tumor microenvironment consists of different types of cells including tumor, immune, stromal, and inflammatory cells all of which produce cytokines, growth factors, and adhesion molecules. All these factors intimately interact with one another and play an important role in inflammatory and pro-angiogenic processes and thereby tumor progression and metastases.

Previous studies have shown that cytokines like IL8 and IL1B are elevated significantly in saliva of OSCC patients.[9,10,11,12] These biomarkers can act as major targets for early detection of cancer. Most of these markers have been identified either in cancer cell lines or in biopsy specimens from cancers. The identification of molecular markers in bodily fluids that would predict the development of cancer in its earliest stage or in precancerous stage would constitute a non-invasive and convenient method. In case of oral cancer, as the neoplastic cells are immersed in the salivary milieu these molecular markers can be detected in saliva conveniently and efficiently.[77]

The aim of our study was to find out if the levels of IL 8 and IL1B are altered in OSCC and OPMD when compared to normal subjects in a South Indian population. Though many studies have been done in Western countries [9,10,11,72,73] very few are done in India[74,45]. Our study population included three groups, group I- OSCC (n=18), group II – OPMD(n=30), group III – normal controls(n=24). Group II was subdivided into group II A – OSF (n= 16) and group II B – leukoplakia (n=14).

Enzyme-linked immunosorbent assay (ELISA) a powerful method for detecting and quantifying a specific protein in a complex mixture used in both experimental and diagnostic techniques. It is a highly sensitive technique that can detect proteins at the picomolar to nanomolar range. This technique was used in our study due to high sensitivity to detect proteins especially low abundant proteins like IL8 and IL1B, affordability of the cost factor and less laboratory demands. We found significant difference in expression of IL 8 and IL 1B in OSCC when compared to controls.

IL8

In our study the concentration of IL 8 was found to be elevated in OSCC when compared to the controls. This is in accordance with the studies which already have proven that IL 8 could be used as a biomarker to detect the presence of OSCC. [9, 10,11,73,74] Though there was a difference between OPMD and controls, it was not statistically significant. We noted an increase in concentration of IL 8 in leukoplakia when compared to OSF which was also not statistically significant. We suggest that comparing more number of samples of leukoplakia with different stages of dysplasia are necessary to understand its significance.

There was no significant difference between the OPMD and controls. This result is consistent with the result of a study by Punyani SR *et al* in 2012[75]. Our study demonstrates the reliability of IL8 as a biomarker for differentiating OSCC from normal. But our study also shows that IL8 cannot be used reliably to differentiate OPMD from normal.

An issue we encountered in IL 8 assay was higher values for standards in IL 8 than given in the data sheet from the provider (Diaclone™, France). This can be attributed to the factors like improper dilution of standards, improper handling during serial dilution, air bubbles in the pipette, touching the base of the wells with the pipette, time factor, and improper calibration of the ELISA reader. As we made sure that we avoided all these factors by proper handling and following the protocol this difference could be attributed to the environmental variation. Moreover the reading was duplicated with similar results and all the concentrations showed a higher absorbance value consistently in repetition. This led us to continue the assay and accept the results.

IL 1B

In the case of IL 1B also the concentrations were found to be significantly elevated in OSCC when compared to controls. This is consistent with the results of studies done by Katakura *et al* in 2007[76] and Brinkman. O *et al* in 2011[9].

In our study on IL1B we encountered a problem of getting higher absorbance than the absorbance of the highest concentration in the standard curve for many samples. This is attributable to many factors like insufficient washing or blocking, sample components or antibodies cross-reacting with the blocking buffer or the use of too much enzyme conjugate, contamination etc. As we have repeated the readings after having ensured that any of these factors did not affect our assay, this could be due to the presence of higher concentration of IL 1B in our study samples beyond the range of that detectable by the technique. IL 1B is an innate immune cytokine which can be secreted by many cells in the oral mucosa. In a study by Kumar PS *et al* smokers

demonstrated significantly greater levels of IL 1 B.[78] It has also been shown that even the commensal oral microflora can induce the secretion of IL1B through Toll-like receptors (TLR) in response to pathogen-associated molecular patterns (PAMPs) or IL-1B itself.[79] It has also been proved that certain bacterial species/ phylotypes may play a role in triggering chronic inflammation in oral cavity and possibly be associated at different stages of cancer in a study by Pushalkar *et al*. [80]

The high absorbance of the samples can also be attributed to the fact that the ELISA kit we used had a low detection range (6 - 500 pg/ml). Previous studies have demonstrated high levels of IL1B which is comparable with our result. Teles *et al* has demonstrated 753 ± 1022 pg/ml of IL1B in saliva of periodontitis subjects in 2009.[81] Moreover IL-1B average levels in a study using single-plex assay by Arellano-Garcia in 2008 were 945.2 ± 1134.8 pg ml−1 (OSCC(n = 20) and 314.2 ± 444.8 pg ml−1 (control, n = 20) which is consistent with our study.[72]

The samples which showed higher levels must ideally have been run in repetitions in different dilutions to get a value within the limit. We suggest that further studies should be done in different dilutions and also using a kit with a wider assay range to substantiate the higher values we got in our study.

In our study we also found that the level of IL 1B in OSCC and leukoplakia (Dysplasia) were similar in saliva. This shows that levels of IL 1B is increased with the presence of dysplastic lesions in the oral cavity when compared to OSF and normal. Further studies needs to be done in a larger sample size with OSCC and lekoplakia stratified based on differentiation and level of dysplasia respectively.

Comparison with group IIIA and B separately

Our study group III consisted of 12 patients with periodontitis (group IIIA) and 12 patients without periodontitis (group IIIB). When IL 8 and IL1B were compared with these subgroups, we found group IIIA and B showed no statistical significance when compared separately with groups I, IIA and IIB. The result we got is not conclusive as our sample size was not sufficient and we suggest validating these results by doing the study with a larger sample size.

Cytokines are intercellular signalling proteins which play a role in regulating growth, cellular proliferation, angiogenesis and tissue repair. They also function in immune responses to infection, injury and inflammation. Some conditions such as rheumatoid arthritis, periodontal disease, osteoporosis, Sjögren's syndrome, chronic parotitis, severe exercise and diabetes can give rise to increased levels of inflammatory proteins. Our control group included patients with periodontal disease (n = 12) and found that while they have slightly elevated IL-8 and IL 1B in saliva, patients with oral cancer have significantly higher saliva levels. This shows that although immunological and inflammatory diseases may increase cytokines levels, the levels are not as high as those found in OSCC patients. But we suggest that local as well as systemic immunological and inflammatory disease control group may be included for further validation of IL-8 and IL-1B as truly discriminatory markers for OSCC.

Summary and Conclusion

- The study group included 18 OSCC (group I), 30 OPMD (16 OSF - group IIA), 14 leukoplakia (group IIB) and 24 controls (group III, 12 with periodontitis (group IIIA and 12 without periodontitis (group IIIB)).
- This study was done to detect and compare the levels of IL8 and IL1B in saliva of patients with OSCC, OPMD and control group
- The technique, solid phase sandwich ELISA was used to identify and quantify the levels of IL8 and IL1B in study groups.
- Mean concentrations of IL8 and IL 1B were assessed in all groups .
- We found that the mean concentrations of both IL 8 and IL 1B were increased in OSCC and OPMD when compared to the control group.
- In multiple comparisons, the concentration of IL 8 was significantly increased in OSCC when compared with OSF ($P<0.05$) and control group ($P<0.05$).
- The concentration of IL 8 in OSCC was increased when compared with leukoplakia ($P=0.14$).
- In multiple comparisons, the concentration of IL1B was increased in OSCC when compared to controls ($P<0.05$).
- The concentration of IL1B in leukoplakia was similar to OSCC ($P=1.0$).
- The concentration of IL1B was increased in OSF ($P=0.83$) as well as leukoplakia ($P=0.64$) when compared to controls.
- When the multiple comparisons were done with groups IIIA and IIIB separately none of the comparisons showed statistical significance.

Salivary diagnostics is an emerging field which has high potential to revolutionize the field of laboratory diagnosis as it is non-invasive and allows easy specimen handling[80]. Our study shows that IL8 and IL1B are significantly elevated in saliva of patients with OSCC when compared to normal controls. This supports the invention of point of care devices for large scale screening for OSCC. More works like this need to be done using a larger sample size, before turning saliva diagnostics into a clinical and commercial reality. Studies like this contribute to the invention and commercialization of lab on chip assays using microfluidics[81] and microbeads which would lead to early detection of serious fatal diseases like oral cancer, assessment of prognosis and treatment outcomes.

Bibliography

1. **World Health Organization**

   The Global Burden of Disease: 2004 Update

   Geneva: World Health Organization, 2008, www cancer org

2. **Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM**

   Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008

   Int J Cancer 2010, 127: 2893–917

3. **Dikshit R , Gupta PC, Ramasundarahettige C ,Gajalakshmi V ,Aleksandrowicz L, Badwe R, Rajesh Kumar R, Roy S, Suraweera W, Bray F, Mallath M, Singh PK, Sinha DN, Shet AS, Gelband H, Jha P**

   Cancer mortality in India: a nationally representative survey

   The Lancet, 2012, 379: 1807 – 1816

4. **Daftary DK, Murti PR, Bhonsle RB**

   Risk factors and risk markers for oral cancer in high incidence areas of the world

   Oral Cancer, Cambridge University Press: Cambridge, 1991, 2: 29-63

5. **Sunny L, Yeole BB, Hakama M, Shiri R, Sastry PS, Mathews S, Advani SH**

   Oral cancers in Mumbai, India: A fifteen years perspective with respect to incidence trend and cumulative risk

   Asian Pac J Cancer Prev 2004, 5: 294-300

6. **Mehrotra R, Yadav S**

   Oral squamous cell carcinoma: Etiology, pathogenesis and prognostic value of genomic alterations

   Indian J Cancer 2006, 43:60-6

7. **Neville B, Damm DD, Allen CM, Bonquot J**

   Oral and maxillofacial pathology

   Saunders publication 2009, 3rd edition

8. **Ranganathan K, Umaadevi M, Joshua E, Kirankumar K, Saraswathi T**

   Oral submucous fibrosis- A case control study in Chennai

   J Oral Pathol Med, 2004:33: 274-7

9. **Brinkmann O, Kastratovic DA**

   Oral squamous cell carcinoma detection by salivary biomarkers in a Serbian population

   Oral Oncol January 2011, 47(1):51-5

10. **Wong DT**

    Salivary Transcriptome Diagnostics for Oral Cancer detection

    Clin Cancer Res 2004, 10: 8442-8450

11. **John MA, Li Y**

    Interleukin 6 and interleukin 8 as potential biomarkers for oral cavity and oropharyngeal squamous cell carcinoma

    Arch Oto laryngol Head Neck Surg 2004, 130(8):929-35

12. **Mantovani A, Muzio M, Garlanda C, Sozzani S, Allavena P**

    Macrophage control of inflammation: negative pathways of regulation of inflammatory cytokines

    Novartis Found Symp 2001, 234:120-31

13. **Dinarello CA, Wolff SM**

    The role of interleukin-1 in disease

    N Engl J Med 1993, 328:106-113

14. **Portier M, Zhang XG, Ursule E, Lees D, Jourdan M, Bataille R, Klein B**

Cytokine gene expression in human multiple myeloma

Br J Haematol 1993, 85:514-520

15. **Elaraj DM, Weinreich DM, Varghese S, Puhlmann M, Hewitt SM, Carroll NM, Feldman ED, Turner EM, Alexander HR**

The role of interleukin 1 in growth and metastasis of human cancer xenografts

Clin Cancer Res 2006, 12:1088-1096

16. **Chen Z, Malhotra PS, Thomas GR, Ondrey FG, Duffey DC, Smith CW, Enamorado I, Yeh NT, Kroog GS, Rudy S, McCullagh L, Mousa S, Quezado M, Herscher LL, Van Waes C**

Expression of pro-inflammatory and pro-angiogenic cytokines in patients with head and neck cancer

Clin Cancer Res 1999, 5:1369-1379

17. **Gemma A, Takenaka K, Hosoya Y, Matuda K, Seike M, Kurimoto F, Ono Y, Uematsu K, Takeda Y, Hibino S, Yoshimura A, Shibuya M, Kudoh S**

Altered expression of several genes in highly metastatic subpopulations of a human pulmonary adenocarcinoma cell line

Eur J Cancer 2001, 37**:**1554-1561

18. **Lewis AM, Varghese S, Xu H and Alexander HR**

Interleukin-1 and cancer progression: the emerging role of interleukin-1 receptor antagonist as a novel therapeutic agent in cancer treatment

Journal of Translational Medicine 2006, 4:48

19. **Voronov E, Shouval DS, Krelin Y, Cagnano E, Benharroch D, Iwakura Y, Dinarello CA, Apte RN**

IL-1 is required for tumor invasiveness and angiogenesis

Proc Natl Acad Sci USA 2003, 100:2645-2650

20. **Waugh DJJ and Wilson C**

The Interleukin-8 Pathway in Cancer

Clin Cancer Res 2008, 14:6735-6741

21. **Newman MG, Takei HH, Klokkevold, PR, Carranza FA**

Carranza's Clinical Periodontology, 10th Edition

Philadelphia: WB Saunders Company, 2006

22. **Navazesh M and Kumar SKS**

Measuring salivary flow: Challenges and opportunities

J Am Dent Assoc 2008, 139:35S-40S

23. **Warnakulasuriya S**

Global epidemiology of oral and oropharyngeal cancer

J oral oncology 2008, 06:002

24. **Sankaranarayanan R, Masuyer E, Swaminathan R, Ferlay J, Whelan S**

Anticancer Head and neck cancer: a global perspective on epidemiology and prognosis

Anticancer Res 1998, 18(6B):477

25. **Khandekar, Bagdey PS, Tiwari RR**

Oral Cancer and Some Epidemiological Factors: A Hospital Based Study S P Indian Journal of Community Medicine 2006, 31: 3

26. **de Souza C, Pawar U, Chaturvedi P**

Precancerous lesions of oral cavity,

Otorhinolaryngology clinics, 2009, 1:7-14

27. **Mhaske S, Ragavendra R , Gupta IMK**

Oral submucous fibrosis - Current Concepts in Etiopathogenesis

People's Journal of Scientific Research 2008, 1: 39-44

28. **Kumar KK, Saraswati TR, Ranganathan K, Uma Devi M, Joshua E**

Oral submucous fibrosis – A clinico-histopathological study in Chennai,

Indian J Dent Res, 2007, 18: 106 -111

29. **Dyavanagoudar SN**

Oral Submucous Fibrosis: Review on Etiopathogenesis

Journal of Cancer Science & Therapy 2009, 1(2) : 072-077

30. **Auluck A, Rosin MP , Zhang H Sumanth KN**

Oral Submucous Fibrosis, a Clinically Benign but Potentially Malignant Disease: Report of 3 Cases and Review of the Literature

JCDA 2008, 74:735-740

31. **Warnakulasuriya S, Johnson NW**

Van der Waal Nomenclature and classification of potentially malignant Disorders of the oral mucosa

J Oral Pathol Med 2007, 36: 575–80

32. **Bouquota JE, Speightb PM, Farthingb PM**

Current Epithelial dysplasia of the oral mucosa—Diagnostic problems and prognostic features

Diagnostic Pathology 2006, 12: 11–21

33. **Napier SS, Speight PM**

Natural history of potentially malignant oral lesions and conditions: an overview of the literature

J Oral Pathol Med 2008, 1:1-10

34. **Hulka BS, Griffith JD, Wilcosky TC**

Overview of biological markers

Biological markers in epidemiology

New York: Oxford University Press, 1990, pp 3–15

35. **Mayeux R**

Biomarkers: Potential Uses and Limitations

The Journal of the American Society for Experimental NeuroTherapeutics April 2004: 1, 182–188

36. **Guyton AC**

Text Book of Medical Physiology, Elsevier Saunders

Philadelphia 11th edition, 2006: 792 -795

37. **Wong DT**

Salivary diagnostics

Wiley-Blackwell USA 1st edition, 2008: 14

38. **Mandel ID**

The role of saliva in maintaining oral homeostasis

J Am Dental Ass 1989, 119:298–304

39. **Hold KM, de Boer D, Zuidema J, Maes RA**

Saliva as an analytical tool in toxicology

Int J Drug Testing, 1995

40. **Dawes C**

Salivary flow patterns and the health of hard and soft oral tissues

JADA 2008, 139:18S-24S

41. **Choo RE, Huestis MA**

Oral fluid as a diagnostic tool

Clin Chem Lab Med 2004, 42(11):1273–1287

42. **Kaufman E and Lamster IB**

The Diagnostic Applications of Saliva-- A Review

Critical Reviews in Oral Biology and Medicine 2002, 13: 197

43. **Lee YH, Wong DT**

Saliva: An emerging biofluid for early detection of diseases

Am J Dent 2009 August , 22(4): 241–248

44. **Lee JM, Garon E, Wong DT**

Saliva Diagnostics

Orthod Craniofac Res 2009 August , 12(3): 206–211

45. **Segal A, Wong DT**

Salivary diagnostics: enhancing disease detection and making medicine better

Eur J Dent Educ 2008 February , 12(Suppl 1): 22–29

46. **Rajkumar K, Kumar AR, Ramyamalini V, Nandhini G, Kumar TD, Aswini BK, Anandan SN**

Estimation of serological and salivary biomarkers with oral squamous cell carcinoma, premalignant lesions and conditions SRM university

journal of dental sciences 2010, 1(1): 14-19

47. **Ghallab NA, El-Wakeel N, Shaker OG**

Levels of Salivary IFN-gamma, TNF-Alfa, and TNF Receptor-2 As PrognosticMarkers in (Erosive) Oral Lichen Planus Mediators of Inflammation Volume 2010, Article ID 847632, 7 pages)

48. **Sashikumar R, Ranganathan K**

Salivary glucose levels and oral candidal carriage in type II diabetics

Oral surg Oral med Oral path and Endodont 2010, 109: 706 – 711

49. **Foley JD, Campbell CL, Miller CS**

ACC Poster Contributions Georgia World Congress Center

JACC March 9, 2010 Volume 55, issue 10A

50. **Hu S, Wang J, Meijer J, Ieong S, Xie Y, Yu T, Zhou H, Henry S, Vissink A, Pijpe J, Kallenberg C, Elashoff D, Loo JA, Wong DT**

Salivary proteomic and genomic biomarkers for primary Sjogren's syndrome Arthritis Rheum 2007, 56: 3588-3600

51. **Ching K H , Burbelo P D , Gonzalez-Begne M**

Salivary anti-Ro60 and anti-Ro52 Antibody Profiles to Diagnose Sjögren's Syndrome J Dent Res 2011, 90: 445-449,

52. **Li C, Ha T, Ferguson DA Jr, Chi DS, Zhao R, Patel NR**

A newly developed PCR assay of H pylori in gastric biopsy, saliva, and feces Evidence of high prevalence of H pylori in saliva supports oral transmission

Dig Di Sci 1996, 41:2142-214

53. **Navazesh M, Kumar SKS**

Measuring salivary flow: Challenges and opportunities

J Am Dent Assoc 2008, 139,35S-40S

54. **Collecting saliva, Salimetrics**

www salimetrics com

55. **Al-Tarawneh SK, Border MB, Dibble CF, Bencharit S**

Defining Salivary Biomarkers Using Mass Spectrometry-Based Proteomics: A Systematic Abstract OMICS

A Journal of Integrative Biology 2011, 15(6): 1-9)

56. **Hu S, Arellano M, Boontheung P, Wang J, Zhou H, Elashoff D, Wei R, Loo JA, WongDT**

Salivary Proteomics for Oral Cancer BiomarkerDiscovery

Clin Cancer Res 2008, 14:6246-6252

57. **de Jong EP, Xie H, Onsongo G, Stone MD, Chen X-B, Kooren JA, Refsland EW, Griffin RJ, Ondrey FG, Wu B, Lee CT, Rhodus NL, Carlis JV, Griffin TJ**

Quantitative Proteomics Reveals Myosin and Actin as Promising Saliva Biomarkers for Distinguishing Pre-Malignant and Malignant Oral Lesions

PLoS ONE 2010, 5(6): 1371

58. **Rao PV, Reddy AP, Lu X, Dasari S, Krishnaprasad A, Biggs E, Roberts CT, Nagalla SR**

Proteomic Identification of Salivary Biomarkers of Type-2 Diabetes

J Proteome Res 2009, 8(1):239-245

59. **Hu S, Wang J, Meijer j, Ieong S, Xie Y, Yu T, Henry S, Vissink A, Pijpe J, Kallenberg C, Elashoff D, Loo JA, Wong DT**

Salivary Proteomic and Genomic Biomarkers for Primary Sjögren's Syndrome. Arthritis Rheum 2007 , 56(11): 3588–3600

60. **Hu S, Li Y, Wang J, Xie Y, Tjon T, Wolinsky L, Loo RRO, Loo JA, Wong DT**

Human Saliva Proteome and Transcriptome

J Dent Res 2006 , 85(12): 1129–1133)

61. **Park NJ, Zhou H, Elashoff D, Henson BS, Kastratovic DA, Abemayor E, Wong DT**

Salivary microRNA: Discovery, Characterization, and Clinical Utility for Oral Cancer Detection

Clin Cancer Res 2009, 15(17): 5473–5477

62. **Hamad AWR, Gaphor SM, Shawagfeh MT Al-Talabani NG**

Study of Serum and Salivary Levels of Proinflammatory Cytokines ,Potential Biomarkers in the Diagnosis of Oral Squamous Cell Carcinoma

Academic Journal of Cancer Research 2011, 4 (2): 47-55

63. **Hofman LF**

Human saliva as a diagnostic specimen

The journal of nutrition (supplement) 2001:01, 1621-1625

64. **Xiao H, Wong D T**

Method development for proteome stabilization in human saliva

Analytica Chimica Acta 722 (2012) 63– 69

65. **Herra SE, Hatcha AV, Giannobileb WV, Throckmortona DJ, Trana HM, Brennana JS**, **Singha JK**

Integrated Microfluidic Platform for Oral Diagnostics

Ann N Y Acad Sci 2007, 1098: 362–374

66. **Elmslie RE, Dow SW, Ogilvie GK**

lnterleukins: Biological Properties and Therapeutic Potential DVM

Journal of Veterinary Internal Medicine 1995, 5(5)

67. **Akdis M, Burgler S, Crameri R, Eiwegger T, Fujita H, Gomez E, Klunker S, Norbert Meyer N, O'Mahony N, Palomares O, Rhyner C, Quaked N, Schaffartzik A, Van De Veen W, Zeller S, Zimmermann M, Akdis CA**

Interleukins, from 1 to 37, and interferon-g: Receptors, functions, and roles in diseases

J allergy clin immunol 2011, 127(3): 701-721

68. **Jin L, Yuan RQ, Fuchs A, Yao Y, Joseph A, Schwall R, Schnitt SJ, Guida A, Hastings HM, Andres J, Turkel, G, Polverini PJ, Goldberg ID, Rosen EM**

Expression of interleukin-1 in human breast carcinoma

Cancer 1997, 80:421-434

69. **Dinarello CA**

Biologic basis for interleukin-1 in disease

Blood 1996, 87:2095-2147

70. **Tsai CC, Chen CC, Lin CC, Chen CH, Lin TS, Shieh TY**

Interleukin-1 beta in oral submucous fibrosis, verrucous hyperplasia and squamous cell carcinoma tissues

Kaohsiung J Med Sci 1999, 15(9):513-9)

71. **Li Y, St John MAR, Zhou X**

Salivary transcriptome diagnostics for oral cancer detection

Clin Cancer Res 2004,10:8442-8450

72. **Arellano-Garcia ME, Hu s, Wang J, Hensen B, Zohu H, Chia A, Wong DT**

Multiplexed immunobead-based assay for detection of oral cancer protein biomarkers in saliva,

Oral Dis 2008, 14(8): 705–712)

73. **Rhodus NL, Ho V, Miller CS, Myers S, Ondrey F**

NF-kappaB dependent cytokine levels in saliva of patients with oral preneoplastic lesions and oral squamous cell carcinoma

Cancer Detect Prev 2005,29(1):42-5

74. **Jamee MS, Eslami M, Moghadam FA, Sarafnejad A**

Salivary concentration of TNFα, IL1α, IL6, and IL8 in oral squamous cell carcinoma

Med Oral Patol Oral Cir Bucal 2008,13(5):E292-5

75. **Punyani SR, Sathawane RS**

Salivary level of interleukin-8 in oral precancer and oral squamous cell carcinoma

Clinical Oral Investigations 2012 © Springer-Verlag

76. **Katakura A, Kamiyara I, Takano N**

Comparison of salivary cytokines in oral cancer patients

Bull Tokyo Dent Coll, 2007, 48: 199-203

77. **El-Naggar AK, Mao L, Staerkel G**

Genetic heterogeneity in saliva from patients with oral squamous carcinomas: implications in molecular diagnosis and screening

J Mol Diagn 2001,3:164 –70

78. **Kumar PS, Matthews CR, Joshi V, de Jager M, Aspiras M**

Tobacco smoking affects bacterial acquisition and colonization in oral biofilms

Infect Immun, 2011

79. **Wells JM, Rossi O, Maejerink M, van Barrien P**

Epithelial crosstalk at the microbiota–mucosal interface

Proceedings of the National Academy of Sciences March 15, 2011 **vol 108 no Supplement 1** ***4607-4614***

80. **Pushalkar S, Xiaojie Ji1 X, Li Y, Estilo C, Yegnanarayana R, Singh B, Li X, Saxena D**

Comparison of oral microbiota in tumor and non-tumor tissues of patients with oral squamous cell carcinoma

BMC Microbiology 2012, 12:144

81. **Teles RP**, **Likhari V**, **Socransky SS**, **Haffajee AD**

Salivary Cytokine Levels in Chronic Periodontitis and Periodontally Healthy Subjects

A cross sectional study

J Periodontal Res 2009 June, 44(3): 411–417

Annexures

ANNEXURE I

DATA SHEET FOR IL 8 ELISA KIT

Intended use

The Diaclone IL-8 kit is a solid phase sandwich ELISA for the *in-vitro* qualitative and quantitative determination of IL-8 in supernatants, buffered solutions, serum and plasma or other biological samples. This assay will recognise both natural and recombinant human IL-8.

Biotinylated anti-IL-8

It is recommended this reagent is prepared immediately before use. Dilute the biotinylated anti-IL-8 with the biotinylated antibody diluent in an appropriate clean glass vial using volumes appropriate to the number of required wells

Assay Step		Details
1.	Addition	**Prepare Standard curve**
2.	Addition	Add 100µl of each, **Sample, Standard, Control and zero (appropriate standard diluent)** in duplicate to appropriate number of wells
3.	Addition	Add 50µl of diluted **biotinylated anti-IL-8** to all wells
4.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25℃) for **1 hour(s)**
5.	Wash	Remove the cover and wash the plate as follows: a) Aspirate the liquid from each well b) Dispense 0.3 ml of **1x washing solution** into each well c) Aspirate the contents of each well d) Repeat step b and c another two times
6.	Addition	Add 100µl of **Streptavidin-HRP** solution into all wells
7.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25℃) for **30 min**
8.	Wash	Repeat wash step 5.
9.	Addition	Add 100µl of ready-to-use **TMB Substrate Solution** into all wells
10.	Incubation	Incubate in the dark for **12-15 minutes*** at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil.
11.	Addition	Add 100µl of **H_2SO_4:Stop Reagent** into all wells
Read the absorbance value of each well (immediately after step 11.) on a spectrophotometer using 450 nm as the primary wavelength and optionally 620 nm as the reference wave length (610 nm to 650 nm is acceptable).		

Data Analysis

Calculate the average absorbance values for each set of duplicate standards, controls and samples.

Generate a linear standard curve by plotting the average absorbance of each standard on the vertical axis versus the corresponding IL-8 standard concentration on the horizontal axis.

The amount of IL-8 in each sample is determined by extrapolating OD values against IL-8 standard

concentrations using the standard curve**.**

Sensitivity - The sensitivity, minimum detectable dose of IL-8 using this Diaclone IL-8 ELISA kit was found to be **29pg/ml**..

Specificity - The assay recognizes both natural and recombinant human IL-8. To define the specificity of this ELISA several proteins were tested for cross reactivity. There was no cross reactivity observed for any protein tested (IL-1a, IL-1b, IL-10 IL-12, IFNg, IL-2, IL-6, TNFa, IL-4 and IL-13).

ANNEXURE II

DATA SHEET FOR IL1B ELISA KIT

Intended use

The Diaclone IL-1B ELISA kit is a solid phase sandwich ELISA for the *in-vitro* qualitative and quantitative determination of IL-1B in supernatants, buffered solutions or serum, plasma and other biological samples. This assay will recognise both natural and recombinant human IL-1B.

Biotinylated anti IL-1B

It is recommended this reagent is prepared immediately before use. Dilute the biotinylated anti IL-1B with the biotinylated antibody diluent in an appropriate clean glass vial using volumes appropriate to the number of required wells

Assay Step		Details
1.	Addition	**Prepare Standard curve**
2.	Addition	Add 100µl of each, **Sample, Standard, Control and zero (standard diluent)** in duplicate to appropriate number of wells
3.	Addition	Add 50µl of diluted **biotinylated anti IL-1β** to all wells
4.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25℃) for **3 hour(s)**
5.	Wash	Remove the cover and wash the plate as follows: a) Aspirate the liquid from each well b) Dispense 0.3 ml of **1x washing solution** into each well c) Aspirate the contents of each well d) Repeat step b and c another two times
6.	Addition	Add 100µl of **Streptavidin-HRP** solution into all wells
7.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25℃) for **30 min**
8.	Wash	Repeat wash step 5.
9.	Addition	Add 100µl of ready-to-use **TMB Substrate Solution** into all wells
10.	Incubation	Incubate in the dark for **10-20 minutes*** at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil.
11.	Addition	Add 100µl of **H_2SO_4:Stop Reagent** into all wells
Read the absorbance value of each well (immediately after step 11.) on a spectrophotometer using 450 nm as the primary wavelength and optionally 620 nm as the reference wave length (610 nm to 650 nm is acceptable).		

Data Analysis

Calculate the average absorbance values for each set of duplicate standards, controls and samples**.** Generate a linear standard curve by plotting the average absorbance of each standard on the vertical axis versus the corresponding IL-1B standard concentration on the horizontal axis. The amount of IL-1B in each sample is determined by extrapolating OD values against IL-1B standard concentrations using the standard curve**.**

Sensitivity

The sensitivity or minimum detectable dose of IL-1B using this Diaclone IL-1B ELISA kit was found to be **6.5 pg/ml**.

Specificity

The assay recognizes both natural and recombinant human IL-1B. To define the specificity of this ELISA several proteins were tested for cross reactivity. There was no cross reactivity observed for any protein tested (IL-1a, IL-2, IL-10, IL-12, IL-17A, IL-23, IFNg, Gp130, IL-33, TNFa).

ANNEXURE -III

From, Date: 26.09.2011

Institutional Review Board,

Ragas Dental College and Hospital,

Uthandi, Chennai

The dissertation topic titled **'Study of interleukin 8 and interleukin 1b in saliva of patients with oral squamous cell carcinoma and oral potentially malignant disorders'** submitted by Dr. Aiswarya Lekshmy.S.U has been approved by the Institutional Review Board of Ragas Dental College and Hospital on 26th September 2011.

Dr.K.Ranganathan

Secretary,

Ragas, IRB

Dr.S.Ramachandran

Chairman,

Ragas, IRB

ANNEXURE: IV

Consent Form

I, …….. s/o, w/o, d/o……., aged about……. years, Hindu/Christian/Muslim/ …….residing at ……

…, do hereby solemnly and state as follows. I am the deponent herein; as such I am aware of the facts stated here under.

I was informed and explained about the pros and cons of the study/test in the …… language known to me.

I was also informed and explained that the results of the individual test will not be revealed to the public. I give my consent after knowing full consequences of the dissertation/thesis/study and I undertake to cooperate with the doctor for the study.

I also authorize the doctor to proceed with further treatment or any other/suitable/alternative method for the study.

I have given voluntary consent to undergo the treatment with out any individual pressure or duress.

I am also aware that I am free to withdraw the consent given at any time during the study in writing.

Signature of the Patient/Attendant

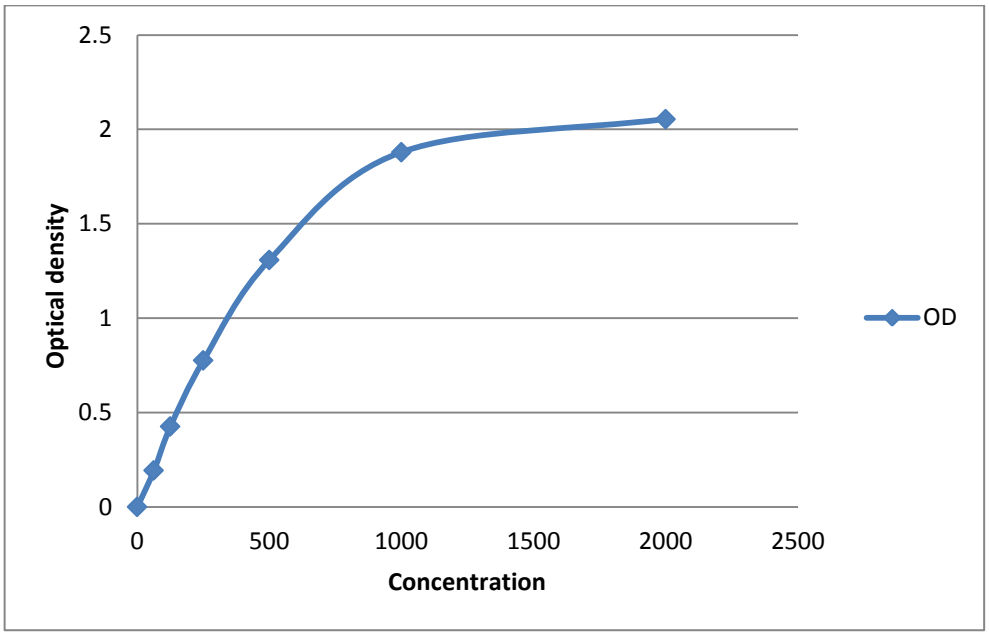
The patient was explained the procedure by me and he has understood the same and signed in

(English/Tamil/Hindi/Telugu/……) before me.

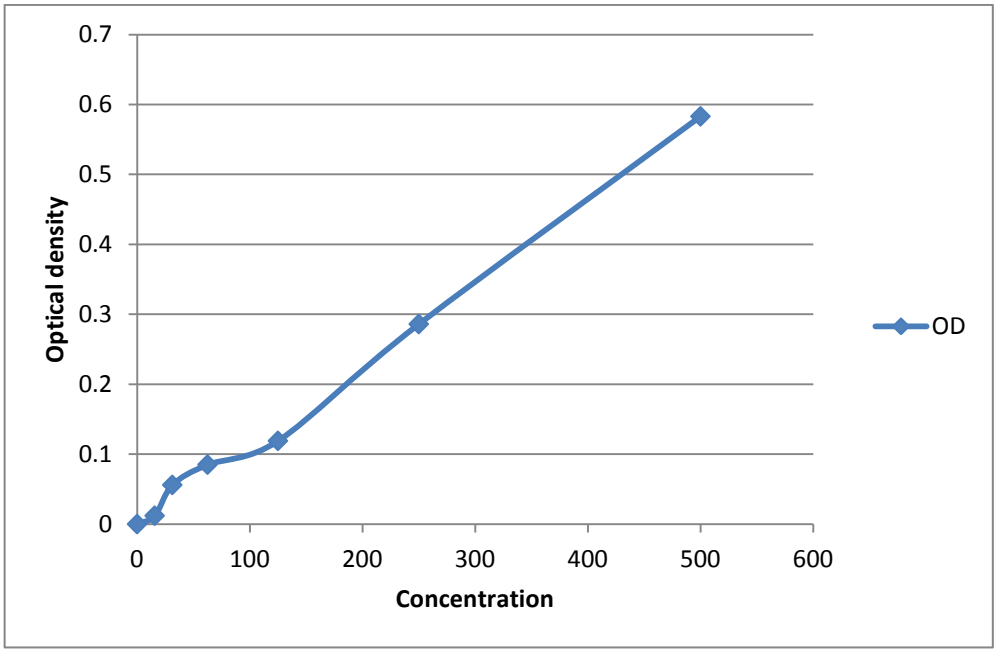
Signature of the Doctor

ANNEXURE: V

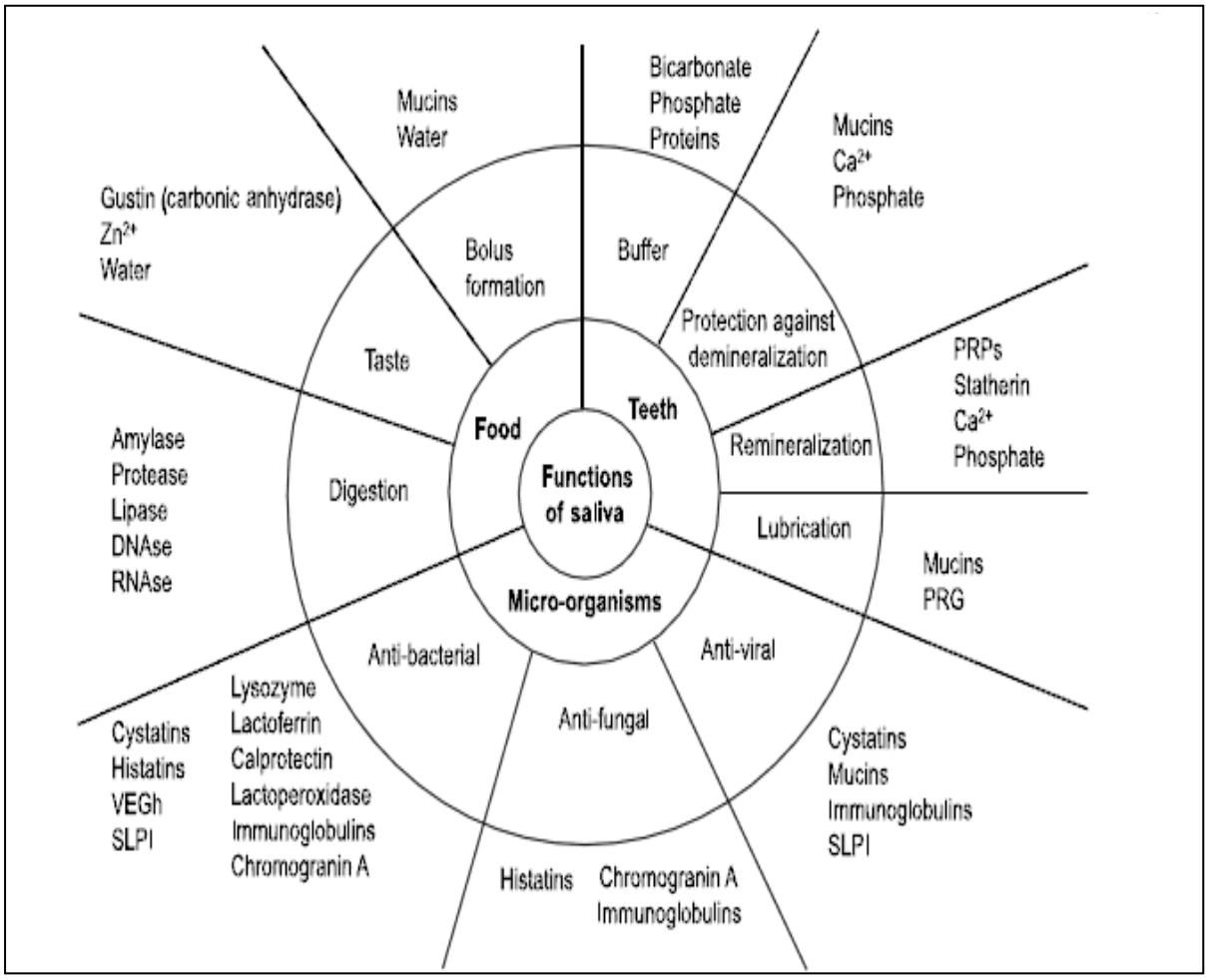
Standard curve with concentrations of IL8 in x-axis and corresponding optical density (OD) in y-axis



Standard curve with concentrations of IL1B in x-axis and corresponding optical density (OD) in y-axis



ANNEXURE: VI



Composition and functions of saliva

Components of saliva and associated functions are depicted in the figure.

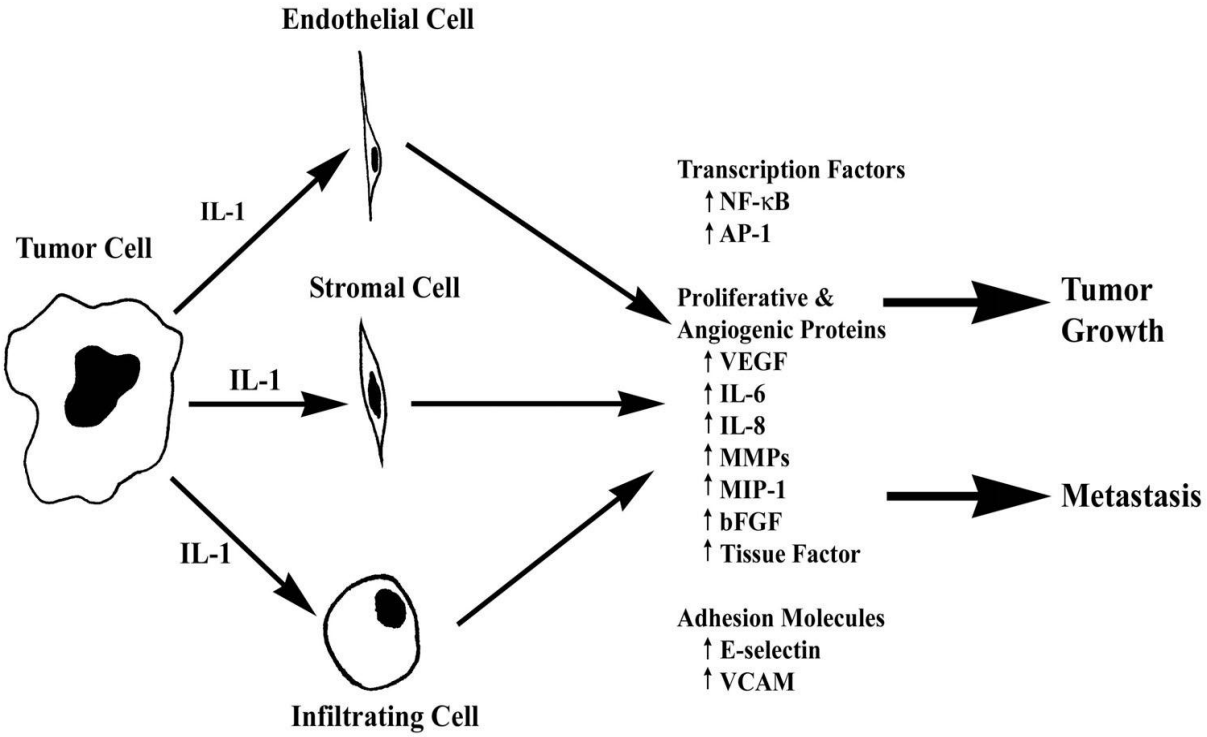
(AV Nieuw Amerongen, ECI Veerman. Saliva - the defender of the oral cavity. Oral Diseases.2002: 8; 12-22)

ANNEXURE: VII

Contributions of different salivary glands to whole saliva volume

Gland	Saliva type	Contribution to volume
Parotid	Serous	25%
Submandibular	Mixed	60%
Sublingual	Mixed	7%-8%,
Minor glands	Mucous	7%-8%

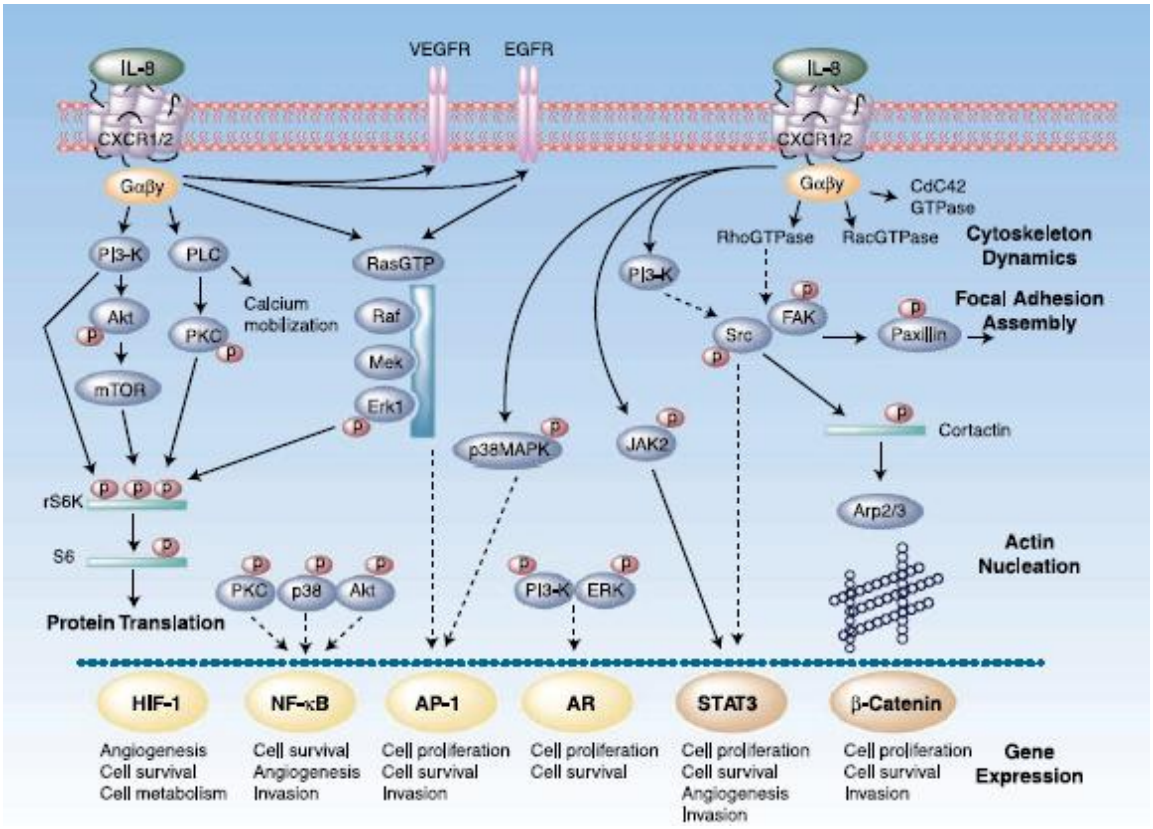
ANNEXURE: VIII



IL-1 indirectly alters tumor growth and metastatic potential

Lewis AM, Varghese S, Xu H, Alexander RH. Interleukin-1 and cancer progression: the emerging role of interleukin-1 receptor antagonist as a novel therapeutic agent in cancer treatment Journal of Translational Medicine 2006, 4:48

ANNEXURE: IX



IL 8 Pathway

Range of signaling pathways that are activated after stimulation of IL8 receptors

(Waugh DJJ and Wilson C. The Interleukin-8 Pathway in Cancer

Clin Cancer Res 2008;14: 6735-6741).

ANNEXURE:XB

MASTER CHART 1 STUDY GROUPS (GROUPI, IIA AND IIB)

SL no	Name	Age	Sex	Othe	Smoki	Num/day	Duration	Chewin	Type	Num/day	Duration	Alchohol	Frequency in ml	Duration	Site	Disease	IL 8	IL1B
1	Achuthan	47	M	hyp	yes	10	15yrs	yes	Panma	10	2yrs	no	no	no	alveolus	OSF/Leu	967	1732
2	Suresh	40	M	no	yes	10	10yrs	yes	manik	2	6yrs	yes	360	10yrs	generaliz	leuk	2070	2081
3	Praveen kur	28	M	no	no	no	no	yes	hans	2pks	5yrs	yes	360	5yrs	generaliz	leuk	747.8	176
4	Manonmani	48	F	insi	no	no	no	yes	manik	20-25p	12yrs	no	no	no	tongue	leuk, dys	1324	1933
5	Akbar ali	31	M	no	yes	5 to 10	3yrs	yes	Panma	5pks	3yrs	no			buccal m	OSF/Leu	1434	116.7
6	Selvam	38	M	no	yes			yes							ton,bucc	leuk	906.5	801
7	Sundar	40	M	no	no			yes	pak	10pks	2yrs	yes	320ml	8yrs	buccal m	leuk, dys	374.1	526
8	Dinakaran	74	M	dial	yes	7-8pks	10yrs	yes				yes			buccal m	OSF/Leu	1120	1349
9	Sampath ku	56	M	no	no			yes	mawa	1pk	20yrs	no			buccal m	leuk	1588	582.9
10	Kuttiyamma	55	F	no	no			yes	tobacc	20 time	30yrs	no			buccal m	leuk	1439	1959
11	Kaliyappan	56	M	no	yes	occa		yes	pak	12-15pk	12yrs	no			tongue	OSF/Leu	955.1	337.8
12	kandasamy	46	M	dial	no			yes	manik	4pks	5yrs	yes	180ml	10yrs	tongue	leuk	0	9.36
13	Antony	43	M	hyp	yes			yes				yes			buccal m	leuk	611.3	672.4
14	Vellaichamy	48	M	no	no			yes	pak	10pks	10yrs	yes	200ml	20yrs	buccal m	oscc	430.6	512
15	Rajendran	39	M	no	yes	4pks	22yrs	yes	pak	10-15pk	3yrs	yes	360ml	3yrs	retromol	oscc	720.7	2215
16	Jayaraman	52	M	An	yes		30yrs	no				yes		30yrs	buccal m	oscc	383.1	727.9
17	Sreedhar	45	M	dial	yes			yes				yes			buccal m	oscc	897	1770
18	Selvin	51	M	no	no			yes	Panma	10pks	25yrs	yes	180ml	28yrs	buccal m	oscc	1596	476
19	Rajesh	27	M	no	yes	2cigs	5yrs	yes	mawa	2pks	12yrs	yes	180ml	5yrs	buccal m	oscc	525.4	726
20	Kuppusamy	50	M	no	yes		3yrs	no				yes			retromol	oscc	1159	290.6
21	Bakul ghosh	37	M	no	no			yes	Panma	conti	22yrs	no			buccal m	oscc	1027	1991
22	Bhaskar	25	M	no	yes			yes				no			buccal m	oscc	1162.11	2201
23	Subramani	48	M	no	no			yes	mawa	4-5 tim	7yrs	no			buccal m	oscc	1198	569
24	Chellappan	62	M	ast	yes	5cigs	5yrs	yes	shanti	10pks	5yrs	yes	180ml	5yrs	buccal m	oscc	977	862
25	Jafer sherif	29	M	no	no			yes	panpar	10pks	3yrs	no			buccal m	oscc	407.2	462
26	Shanmugha	44	M	no	no			yes	Hans	2pks	10yrs	yes	720ml	5yrs	generaliz	osf	73	236
27	Govindraj	33	M	no	no			yes	mawa	2pks	2yrs	yes	720ml	4yrs	generaliz	osf	183	89
28	Lawrence	36	M	no	no			yes	pan	4tim 1p	1yr	yes	270ml	2yrs	generaliz	osf	453	261
29	Jayapal	28	M	no	nn			yes	hans	1pk	5yrs	no			buccal m	oscc	674.1	799
30	Jayalakshm	65	F	dial	no			no				no			alveolus	oscc	996.1	622
31	Rajesh	28	M	no	yes	2cigs	5yrs	yes	mawa	2pks	12yrs	yes	180ml	5yrs	buccal m	osf	51.3	49.09
32	Jagannatha	83	M	no	no			yes				no			alveolus	osf	1132	1401
33	Dhanasekha	37	M	no	yes	20cigs	1yr	yes	gutka		2yrs	yes	380ml	3yrs	generaliz	osf	85.95	47.69
34	Ramanad	52	M	no	no			yes	gutka		30yrs	no			buccal m	osf	74.62	84.25
35	Purushotha	42	M	gas	no			yes	mawa	3-4pks	12yrs	no			buccal m	oscc	1420	1104
36	Elumalai	37	M	TB	no			yes	mawa	10pks	20yrs	no			retromol	osf	1227	519.2
37	Palchamy	74	M	dial	no			yes	pan, manik		>50yrs	yes		15yrs	alveolus	osf	1522	1695
38	Sivakumar	36	M	no	no			yes	mawa	3-4pks	1yr	yes			alveolus	osf	856	638.2
39	Dakshinamu	67	M	no	no			yes	pan			no			buccal m	leuk	807.2	179
40	Raja	40	M	no	no			yes	mawa	4pks	8yrs	yes	360ml	10yrs	buccal m	osf	1231	1905
41	Rajaprasanr	41	M	dial	no			yes	manik	1pk, on	6months	yes	360ml	6months	buccal m	leuk	90.21	109
42	Ramesh	36	M	no	no			yes	mawa,	1pk eac	13yrs	yes	720ml	10yrs	buccal m	osf	356.4	781.9
43	Shivakumar	33	M	no	no	no		yes	mawa	3pks	3yra	yes	720ml	15yrs	buccal m	osf	975.6	1760
44	Vijayakumar	34	M	no	yes	3cigs	5yrs	yes	mawa	1pk 3 ti	1yr	yes	720ml	2yrs	buccal m	leuk	801.2	1156
45	ravindran	46	M	no	yes	1pk	25yrs	no				yes	720ml	25yrs	buccal m	leuk	1018	2080
46	Iqbal	32	M	no	yes			no				yes	720ml	7yrs	buccal m	leuk	59.82	95.44
47	Saravanan	36	M	hyp	no			yes	panpar	4pks	7yrs	yes			tongue	oscc	156.5	64.8
48	Jagannatha	60	M	dial	yes			yes				yes			alveolus	oscc	1204	2057

ANNEXURE:XB

MASTER CHART II CONTROL GROUP (GROUP IIIA AND B)

Case no	Sample no	Age	Gender	Periodontitis	MH	Habits	Il 8	Il 1b
1	CP001	40	F	yes	no	no	0	353.4
2	CP002	38	F	yes	no	no	344	24.99
3	CP003	55	F	yes	hypr	no	1332	193.7
4	CP004	50	F	yes	no	no	0	581.5
5	CP005	52	M	yes	diab	Smoking	54.53	0
6	CP006	35	M	yes	no	no	1297	19.02
7	CP007	42	M	yes	no	no	1420	196.4
8	CP008	35	M	yes	no	no	741.9	972.11
9	CP009	35	M	yes	no	no	968.1	1529
10	CP010	43	F	yes	no	no	339.5	584.6
11	CP011	31	F	yes	no	no	1105	478.3
12	CP012	46	M	yes	no	no	NO	74.83
13	CN001	29	M	no	no	no	957.4	137.2
14	CN002	28	F	no	no	no	157.2	164.4
15	CN003	29	F	no	no	no	1215.1	1002
16	CN004	40	M	no	no	no	1332	-4.88
17	CN005	30	M	no	no	no	0	25.23
18	CN006	25	M	no	no	no	472	1421
19	CN007	27	F	no	no	no	787	745.9
20	CN008	28	F	no	no	no	871.1	1232
21	CN009	35	M	no	no	no	425.3	163.5
22	CN010	31	F	no	no	no	460.4	662.7
23	CN011	45	M	no	no	no	688.3	718.7
24	CN012	42	M	no	no	no	1074	1180

MH - Medical History

hyp - hypertension

diab - diabeties