ESTIMATION OF SALIVARY SIALIC ACID IN ORAL PREMALIGNANCY AND ORAL SQUAMOUS CELL CARCINOMA

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BRANCH IX

ORAL MEDICINE AND RADIOLOGY

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CHENNAI

DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation titled " **ESTIMATION OF SALIVARY SIALIC ACID IN ORAL PREMALIGNANCY AND ORAL SQUAMOUS CELL CARCINOMA** " is a bonafide and genuine research work carried out by me under the guidance of **Dr. S. KAILASAM, B.Sc., M.D.S.**, Professor and Head, Department of Oral Medicine & Radiology, Ragas Dental College and Hospital, Chennai.

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CERTIFICATE

This is to certify that this dissertation titled **"ESTIMATION OF SALIVARY SIALIC ACID IN ORAL PREMALIGNANCY AND ORAL SQUAMOUS CELL CARCINOMA"** is a bonafide record of work done by **Dr. S. EZHIL PALLAVI** under my guidance during her postgraduate study period 2016 – 2020.

This dissertation is submitted to **THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY,** in partial fulfilment for the degree of **MASTER OF DENTAL SURGERY, BRANCH IX – Oral Medicine & Radiology.**

It has not been submitted (partial or full) for the award of any other degree or diploma.

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LIST OF ABBREVIATION

S.NO	ABBREVIATION	EXPANSION
1	WHO	World Health Organization
2	PMD	Potentially Malignant Disorders
3	OSMF	Oral Submucous Fibrosis
4	OSCC	Oral Squamous Cell Carcinoma
5	TSN	Tobacco-Specific Nitrosamines
6	GST	Glutathione-S-Transferase
7	DNA	Deoxyribose Nucleic Acid
8	WDSCC	Well-Differentiated Squamous Cell Carcinoma
9	MDSCC	Moderately-Differentiated Squamous Cell Carcinoma
10	PDSCC	Poorly-Differentiated Squamous Cell Carcinoma
11	ELISA	Enzyme Linked Immunosorbent Assay
12	FSA	Free Sialic Acid
13	PBSA	Protein Bound Sialic Acid
14	Streptavidin – HRP	Streptavidin- Horse Radish Peroxidase

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Introduction

INTRODUCTION

Oral cancer is a major threat to public health. It has a high mortality rate and it is the sixth common cancer in humans in the world [1]. India accounts for about one-third of the world burden of oral cancer. In males, oral cavity is the fourth most common site for cancer and in females it is the fifth most prevailing site after cervix, breast, stomach and liver. About 50-70% of cancer related deaths in India are due to oral cancers.

The etiological factors ascribed to this are habit of using tobacco in any form either smoking/smokeless, alcohol consumption, nutritional deficiency and other disparate factors. The data from WHO global oral health programme suggested that tobacco use & excessive alcohol consumption was estimated to account for about 90% of oral cancers [2]. Hereditary factors & viral infections along with life style dietary factors can also contribute to the etiology of oral cancer.

In the oral cavity, squamous cell carcinoma is the most prevalent with a high morbidity and mortality rate [3]. India has the highest incidence of OSCC in the world. Oral cancer is usually preceded by oral precancerous lesions & conditions (now named as potentially malignant disorders (PMD)) such as oral leukoplakia, OSMF with a malignant transformation rate from 0.6 to 3.6%. Also, appreciation and diagnosis of early stage of oral cancer is the most difficult task. It usually remains asymptomatic for a long period of time and by the time patient seeks

medical advice it would have deeply invaded the tissues and hence prognosis become poor. The present clinical ways to deal with cancer diagnosis and treatment involves invasive & painful procedures. Therefore, a noninvasive procedure using a tumor marker is essential for early diagnosis of oral cancer as well as to monitor its progression during treatment.

Tumor markers are biochemical substances elaborated by tumor cells into the circulation due to aberrant glycosylation that occur as an effect of carcinogenesis[4]. Once such substance is SIALIC ACID (N-ACETYL NEURAMINIC ACID). It is a nine-carbon monosaccharide which is negatively charged and linked to the non-reducing residues of the carbohydrate chain of glycoconjugates by a glyosidic linkage. It is important in determining the surface properties of cells and hence play a valid role in cellular adhesiveness, invasiveness and immunogenicity [5].

Saliva being a biofluid which is a filter of serum from vasculature that nourishes the salivary glands. Any change that occurs in the serum as a result of disease process will be reflected in saliva. The other most important point for selecting saliva as a diagnostic tool is that it also contains the fallen cells in oral cavity which allow saliva to be the first choice of screening and identification of potential biomarkers in the oral cancer [6].

Unfortunately, salivary analysis is not given due thrust in India. There is paucity of studies on analysis of salivary glycoconjugates in cancer. Because of its

proximity to oral neoplasms & premalignant lesions, saliva could be the ideal tool for screening, diagnosis & management of oral cancers [7]. So, this present study is to evaluate and correlate the levels of salivary sialic aid in patients with OSCC and oral premalignancy with those of healthy controls.

Aims & Objectives

AIMS AND OBJECTIVES

AIM OF THE STUDY:

To estimate and correlate the salivary levels of sialic acid in patients with OSCC and oral premalignancy with those of healthy subjects.

OBJECTIVE OF THE STUDY:

- The purpose of the study was to assess the salivary levels of sialic acid in study group comprising of patients with OSCC and oral potentially malignant disorders and healthy control group.

- To compare and correlate the salivary sialic acid levels of patients with OSCC and oral potentially malignant disorders with those of healthy control group.

- To evaluate the changes in the salivary sialic acid levels in the various histopathological grades of OSCC.

Review of literature

REVIEW OF LITERATURE

This study is about estimation of salivary levels of sialic acid in patients with oral squamous cell carcinoma and comparison of the same with patients with oral potentially malignant disorders and healthy controls. A detailed review of literature will accentuate the importance of the study and also concise about different aspects of oral cancer and obtains an interrelationship of the glycoprotein sialic acid in saliva in patients with oral squamous cell carcinoma, potentially malignant disorders and healthy controls.

The frequency of cancer in the head and neck region atone for 30% – 40% of all malignant tumours in India. In males, oral cavity is the fourth most common site for cancer after lungs, stomach and liver and in females it is the fifth most prevailing site after cervix, breast, stomach and liver. In men, a total of 222,000 new cases and in women 90,000 new cases are diagnosed every year worldwide. It was found that about an average of 4.9 million people died of tobacco-related illness in the year 2000, and by 2020s it will increase to 10 million deaths per year [8].

Oral cancer refers to all malignancies arising from oral cavity, lips and pharynx and it affects more than 481,000 new patients worldwide. It is the most common cause for cancer-related deaths among Indian men, which is usually preceded by oral potentially malignant disorders most often a persistent leukoplakia or oral submucous fibrosis (SMF).

Oral leukoplakia and SMF have been reported to show an increased risk of conversion to malignant transformation varying from 0.13% to 6%, and the risk further increased to 14% or higher in dysplastic lesion. Most of the cancers in oral cavity are oral squamous cell carcinoma (OSCC).

Shpitzer T et al. [3] (2007) documented that oral squamous cell carcinoma (OSCC) is the most prevalent cancer in the oral cavity with a high morbidity and mortality rate.

The most common risk factors blamed in the aetiology of oral cancer are use of tobacco (both smokeless and chewing forms), alcohol, irritation, ultraviolet radiation, free radicals, viruses, family history, candidiasis, diabetes. It is well established that there is a strong association between cancers of oral cavity and pharynx with constant use of tobacco. Many studies have shown that the risk

of developing oral cancer is greater for smokers than for non- smokers and this risk may increase even greater for extremely heavy smokers.

Benz-pyrene and tobacco-specific nitrosamines (TSNs) namely 4-(nitroso methylamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosonornicotine (NNN) are the carcinogens present in tobacco. This TSNs covalently bind with deoxyribonucleic acid (DNA) of keratinocytes to form DNA adducts. These DNA adducts are responsible for mutations involved in DNA replication. The metabolism of these carcinogens involves oxygenation by cytochrome P450 enzymes and conjugation by glutathione-S-transferase (GST) [9]. Genetic polymorphisms in the genes coding for these enzymes are suspected to play an important role in the genetic predisposition to tobacco-related head and neck cancers [10].

The use of tobacco in smokeless form has become common globally. This form of tobacco is placed in contact with the mucous membranes inside the oral cavity. The nicotine present in the tobacco is absorbed via the mucosa to provide the desired effect. They have been used in many forms in different parts of the world. For example, use of oral snuff powder is more common in the Middle East and West. Also, betel quid chewing in different forms and with various ingredients is common in Asia, where it is a custom and cultural

habit since tobacco reached India via the Portuguese, who brought it to Europe and Asia from South America [9].

ALCOHOL – tends to increase the risk of development of oral cancer by acting synergistically with tobacco [9].

Keller AZ et al [11] (1969) in his study documented that the role of alcohol in development of oral epithelial dysplasia is pivotal only when it is used along with tobacco.

Jafarey NA et al [12] (1977) documented that, alcohol acts as an independent risk factor for oral leukoplakia in Indian population.

In 1978, WHO proposed the terms "precancerous conditions" and "precancerous lesion"

PRECANCEROUS LESION is defined as "tissue with morphological alterations which has increased risk of developing cancer than its apparently normal counterpart".

PRECANCEROUS CONDITIONS are defined as a generalized state that is associated with a significantly increased risk for cancer development.

The World health organisation in the year 2005 recommended abandoning this terminology and instead proposed to use the term "oral potentially malignant disorders" (OPMD) [13].

OPMD is defined as

> The risk of malignancy being present in a lesion or condition either at the time of initial diagnosis or at a future date."

Oral potentially malignant disorders (OPMD) now a days became more common in general population because of increased habit of using tobacco either in smoking /smokeless form and they are important target for cancer prevention. Most of the OSCC develop from oral potentially malignant disorders. OPMD that have been incriminated in the development of oral cancer include leukoplakia, palatal lesions of reverse smoking, erosive form of lichen planus, oral submucous fibrosis, hereditary disorders such as dyskeratosis congenita. Early detection of OPMD is of utmost importance to

prevent further morbidity as they have an increased risk of cancer transformation.

Potentially Malignant Disorders (PMDs)

Premalignant lesions	Premalignant conditions
Leukoplakia	Lichen planus
Erythroplakia	Discoid lupus erythematosus
Proliferative verrucous leukoplakia(PVL)	Epidermolysis bullosa
Viadent leukoplakia	Verruciform xanthoma
Candida leukoplakia	Graft-versus-host-disease
Reverse smokings' palate	Cheilitis glandularis
Verrucous hyperplasia	Xeroderma pigmentosum
Oral verrucous carcinoma	Syphilis (third stage)
Dyskeratosis congenita	Plummer-Vinson syndrome
Actinic cheilosis	Malnutrition
Keratoacanthoma	Vitamin A, B, C deficiency
Oral submucous fibrosis	Immunosuppressive diseases [AIDS]

Jay Gopal Ray et al [13] (2017) – suggested the most common OPMD lesions are oral leucoplakia, OSMF, erythroplakia and verrucous carcinoma. Some miscellaneous inherited/acquired diseases such as xeroderma pigmentosum, dyskeratosis congenita, Fanconi's anemia, chronic iron deficiency anemia and immunodeficiency are the other potentially malignant disorders for oral carcinoma.

The competent way to manage oral cancer is to combine early diagnosis and timely and appropriate treatment. Because most of the oral cancers are squamous cell carcinomas, the vast majority of oral cancers will be diagnosed from lesions on the mucosal surfaces.

DIAGNOSIS OF ORAL CANCER –

The dentists challenge lies in differentiating cancerous lesions from a multitude of other white, red, speckled or ulcerated lesions that also occur in the oral cavity. Although many of the oral lesions are benign, many have an appearance that could be confused with a malignant lesion, and some previously considered benign are now classified premalignant because they have been statistically correlated with subsequent cancerous changes. On the other hand, some malignant lesions seen in an early stage may be mistaken for a benign change. So, it is mandatory to follow up any suspected lesion and if the oral lesion does not regress spontaneously or respond to the usual therapeutic measures, they should be considered potentially malignant until histopathology proves it as benign. The lesion is usually followed up for a period of 2 – 3 weeks which is considered as an appropriate time period to evaluate the response of a lesion to therapy before obtaining a definitive diagnosis.

To obtain a definitive diagnosis biopsy is mandatory which is obtained using surgical scalpels or biopsy punches and are usually performed under local anaesthesia. In case of incisional biopsy, a representative sample of the lesion is removed whereas in excisional biopsy complete removal of the lesion is done with a border of normal tissue. In few cases multiple biopsies may be required to define the extent of the primary disease and to evaluate the patient for the presence of possible synchronous second malignancies. They are many useful adjuncts to biopsies which include vital staining, exfoliative cytology, fine needle aspiration biopsy, routine dental radiographs and other plain films, and imaging with magnetic resonance imaging (MRI) or computed tomography (CT)

SALIVARY BIOMARKERS FOR OSCC –

SALIVA -

Saliva is an extracellular fluid produced and secreted by salivary glands in the mouth, comprising 99.5% of water and 0.5% of organic and inorganic constituents. Inorganic elements consist of minerals such as sodium, potassium, calcium, magnesium, fluoride, and phosphates. Organic elements comprise enzymes, hormones, immunoglobulins, proteins antioxidants, and coagulation factors are present in the saliva. These constituents are present in the saliva at a varying proportion and which tends to vary during oral cancer.

Many of these constituents, their altered levels in oral cancer can serve as the potential biomarker and aid in its diagnosis [6].

Jiang J et al [14] (2009) documented that saliva is an informative body fluid containing DNA, mRNA and proteins that can be used as biomarkers for translation and clinical applications.

ADVANTAGES OF SALIVA AS A DIAGNOSTIC TOOL –

Simplicity of collection, non-invasive procedure, cost-effectiveness, easy availability of large sample volume for analysis and repeated sampling for monitoring over time [7].

Thus, the saliva-based analysis serves as a non-invasive alternative to serum analysis for diagnosis and prognostication of cancer as well as for monitoring post-treatment therapeutic response of the patients.

Among all the malignancies, oral cancer is one such malignancy, where the saliva examination for detection shows the greatest benefit because of its direct contact with oral cancer lesions. The most important point for selecting saliva as a diagnostic tool is that it also contains the fallen cells in oral cavity

which allow saliva to be the first choice of screening and identification of potential biomarkers in the oral cancer.

Furthermore, health-care experts prefer a salivary test than using serum, because the latter is more likely to expose the technicians to various blood-borne diseases. Although, presence of sialic acid in saliva is low when compared to that of serum, detectable amount of sialic acid can be found in subjects with OPMD and oral cancer when compared to normal subjects.

TUMOR MARKER FOR OSCC –

Lehto and Pontén (1989) - defined tumour markers as

> Specific structurally altered cellular macromolecules or spatially or quantitatively altered normal molecules that are associated with malignant neoplastic cells [4].

They are endogenous products that are produced in the cancer cells at a greater rate than normal cells or the products of newly switched on genes that remain dormant in the normal cells.

The tumour markers are present either as released substances in the circulating body fluids such as serum, urine, cerebrospinal fluid, and saliva or as intracellular substances in tissues. Examples of using body fluids for tumour detection include sputum for the lung cancer diagnosis, urine for the urologic tumours, saliva for the OSCC as well as serum or plasma for almost all types of cancers.

Achalli et al [15] (2017) documented that tumour markers are substances that are specific for certain cancerous cells which serves as an essential tool for diagnostic and prognostic purpose in oral cancer patients.

Kaplan and Pesce [4] (2003) have suggested the following criteria for an ideal tumour marker:

- ✓ Be easy and inexpensive to measure in readily available body fluids
- ✓ Be specific to the tumour being studied and associated with it
- ✓ Have a stoichiometric relationship between plasma levels of the marker and the associated tumour mass
- ✓ Have an abnormal plasma level, urine level, or both in the presence of micro-metastases, that is, at a stage when no clinical or presently available diagnostic methods reveal their presence

- ✓ Have plasma levels, urine levels, or both that are stable and not subjected to wild fluctuations
- ✓ They should prognosticate a higher or lower risk for eventual development of recurrence
- ✓ They should change as the current status of the tumour changes over time
- ✓ They should precede and predict the recurrences, before they are clinically detectable.

Joshi M et al [16] (2010) documented that during tumour development substances that change quantitatively in the serum are collectively called biochemical serum markers or tumour markers.

Thus, these products that are rendered by cancer cells could be of diagnostic and prognostic value in cancer patients. Selected glycolipids and glycoproteins serve as tumour markers because these are the main constituents of the cell membrane. Increase in the levels of glycopeptide containing sialic acid, mannose, fucose and galactose are found in various types of cancer transformed cells and solid tumours, highlighting the association between malignant transformation and changes in the cell-surface glycoconjugates.

Raval GN & Patel PS et al [17] (2003) in his study revealed that the most important molecular change that accompany malignant transformation is the altered glycosylation of glycoconjugates and one such glycopeptide is sialic acid (N- acetyl neuraminic acid), which serve as a tumour marker for OSCC.

Shantaram M et al [18] (2009) revealed that cell surface glycoconjugates are considered to be important in relation to cancer because many of the altered properties of cancer cells are expressed at the surface of the cells.

Sialic acid (N- acetyl neuraminic acid) occurs as terminal component at the nonreducing end of carbohydrate chains of glycopeptide. It determines the surface properties of cells and has been involved in cellular adhesiveness, immunogenicity and invasiveness.

Sialic acid is a protein-bound monosaccharide which occurs in combination with other mono-saccharides like galactose, mannose, glucosamine and fucose. Its level in body fluids is studied for the assessment of synthesis and secretion of glycoprotein.

FIG 1: CHEMICAL STRUCTURE OF SIALIC ACID

They are found as components of both external and internal membrane areas where they are well exposed and develop vital functions. Serum and salivary levels of sialic acid has been known to increase in patients with various types of malignancies and also in conditions like acute inflammation, rheumatoid arthritis and high fever.

The universal feature of cancer is aberrant glycosylation and the levels of these glycoconjugates increases as the cancer development progress. Because of overexpression of sialyl transferases there will be increase in sialylation in cancer.

In oral cancer and oral potentially malignant disorders (OPMDs), there is high cell turnover rate and shedding of malignant cells which increases the sialic acid level, which in turn is released into the circulation

Baxi BR and Patel PS et al [19] (1990) documented that sialic acid plays a vital role in cell-cell recognition, invasiveness, adhesiveness and immunogenicity.

Baxi BR et al [20] (1991) in his study found that serum levels of sialic acid were markedly increased in patients with OSCC and OPMD when compared to that of healthy controls. His study also revealed that patients with metastasis had increased levels of sialic acid than patients with OPMD. The study also obtained a similar result in saliva, although the study failed to determine the interrelationship of salivary levels of sialic aid with metastatic lesions.

Rajpura et al. [21] (2005) estimated and correlated the serum levels of sialic acid in patients with oral potentially malignant disorders, OSCC and healthy subjects and found that serum sialic acid levels were elevated in untreated OSCC patients when compared to that of healthy individuals and patients with oral potentially malignant disorders. He also revealed that there is progressive rise in sialic acid with the stage of the malignant disease.

Sanjay et al [22] (2008) in his study reported an increased level of salivary sialic acid, total protein and total sugar in patients with OSCC when compared to that of healthy individuals.

Joshi M et al [16] (2010) documented that among the glycoconjugates, sialic acid is present up to 30% in various glycoproteins and are expressed on the surface of the cells.

The study conducted by **Dhakar et al [23] (2013)** showed a significant increase in serum and salivary levels of sialic acid and serum protein from healthy controls to OSCC and documented that these markers may be trustworthy in diagnosis and in predicting treatment response and prognosis. He also showed that there is increasing trends in salivary sialic acid levels with increasing levels of histopathological grades of OSCC.

Hemalatha et al [24] (2013) in his study estimated salivary levels of sialic acid in patients with different clinicopathological stages of oral leukoplakia and OSCC and documented that there was marked increase in both free and protein bound sialic acid in saliva of patients with OSCC when compared to patients with leukoplakia.

Vajaria et al [25] (2013) documented that salivary levels of sialic acid were increased in oral cancer and OPMD because of altered sialylation in saliva.

Chaudhari et al [26] (2016) in his study, evaluated and correlated the salivary sialic acid levels among study group comprising of OSCC, PMD patients and control group comprising of healthy subjects and documented that there was a statistically significant difference between the two groups (i.e) the mean salivary levels of sialic aid were found to be increased in malignant as compared to that of premalignant followed by the control group. He also documented that there is progressive increase in salivary sialic acid levels in various histopathological grades of OSCC (i.e) there is increase in salivary sialic acid levels in grade III (poorly differentiated) followed by grade II (moderately differentiated) and grade I (well differentiated).

Achalli et al [15] (2017) in his study documented that glycoproteins also form an essential constituent of salivary mucins. So, altered sialylation in cancer cause increase in sialic acid level in saliva also. He showed that there is progressive increase in salivary levels of sialic acid from controls to OPMD to OSCC.

Poudel et al [27] (2019) in his study showed that there is increased level of salivary sialic acid in oral cancer patients when compared to that of control

group. He also showed that there is significant increase in salivary sialic acid levels in PDSCC followed by MDSCC and WDSCC.

Materials and Methods

MATERIALS AND METHODS

This is a hospital-based study designed to estimate the salivary levels of sialic acid in study group comprising of patients with oral squamous cell carcinoma, oral potentially malignant disorders and to compare the same with control group comprising of healthy subjects and between various histopathological grades of OSCC. Patients were selected from Department of Oral Medicine and Radiology, Ragas Dental College and Hospital, Uthandi, Chennai & Dr. Rai Memorial Cancer institute, Teynampet, Chennai.

STUDY DESIGN: Prospective Study

STUDY PERIOD: This study was done from February 2019 to August 2019

STUDY SETTING :

- Department of Oral Medicine, Diagnosis and Radiology, Ragas Dental College and Hospital, Chennai-600119.
- Dr. Rai memorial cancer institute, Teynampet, Chennai.
- Aara laboratories, Roypettah.

STUDY GROUPS:

- **GROUP I:** 20 subjects with clinically and histopathologically confirmed oral squamous cell carcinoma
- **Group II:** 20 subjects with clinically and histopathologically confirmed oral potentially malignant disorders.
- **Group III:** 20 normal healthy controls without habit of taking tobacco in any form

ETHICAL CONSENT:

This study was approved by institutional ethical committee of Ragas Dental College and Hospital, Chennai. Also, consent was obtained from all the subjects participating in the study in Tamil and English

SELECTION CRITERIA:

Sixty subjects were included in the study. The subjects where divided into 3 groups

INCLUSION CRITERIA:

GROUP I: Patients with clinically and histopathologically confirmed oral squamous cell carcinoma

GROUP II: Patients with clinically and histopathologically confirmed oral potentially malignant disorders such as leukoplakia, OSMF without coexisting OSCC

GROUP III: Normal healthy subjects as controls

EXCLUSION CRITERIA:

GROUP I:

Oral squamous cell carcinoma patients associated with other conditions which include patients with Liver disease, Diabetes mellitus, Renal disease, Co-existing tumors e.g. Brain tumors, Carcinoma of breast, lungs etc.

GROUP II:

Oral potentially malignant disorder patients with history of systemic illness like liver or kidney disease, diabetes, hypertension, cardiovascular disease, depression, OSCC, hyperthyroidism, obesity or sepsis.

GROUP III:

- Healthy volunteers with any habit of using tobacco either in smoking / smokeless form

- Pregnant women

PROCEDURE FOR EXAMINATION:

A proper clinical history was obtained from all the subjects participating in the study. All the subjects were explained about the study and written consent in both English and their local languages was obtained. After a thorough clinical examination, a clinical diagnosis was made. The clinical diagnosis was further confirmed by histopathological examination and final diagnosis was obtained. Patients diagnosed with OSCC were further divided into 3 groups as well differentiated, moderately differentiated and poorly differentiated OSCC based on Bryne's histopathological grading system [28].

CLINICAL EVALUATION –

Armamentarium used for clinical evaluations:

- Dental chair with light source
- Kidney tray
- Mouth mirror

- Probe
- Tweezer
- Disposable gloves
- Disposable mask
- Cotton
- Gauze pieces
- Sterile container with readings in ml

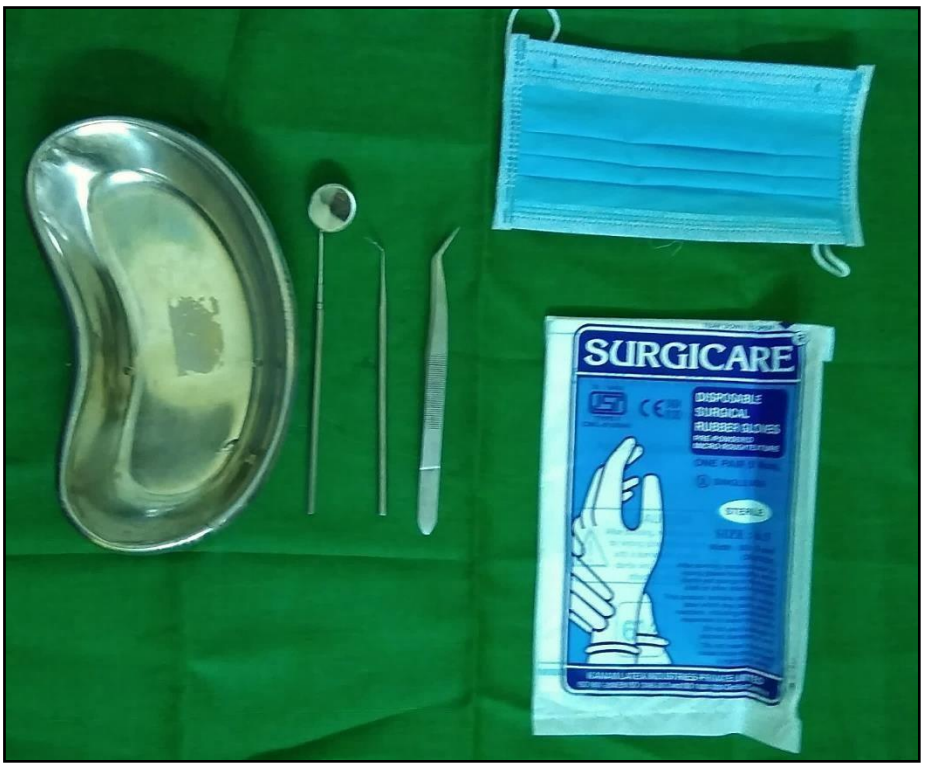


FIG 2: Armamentarium for clinical examination

Histopathological grading of OSCC was done based on Bryne's grading system –

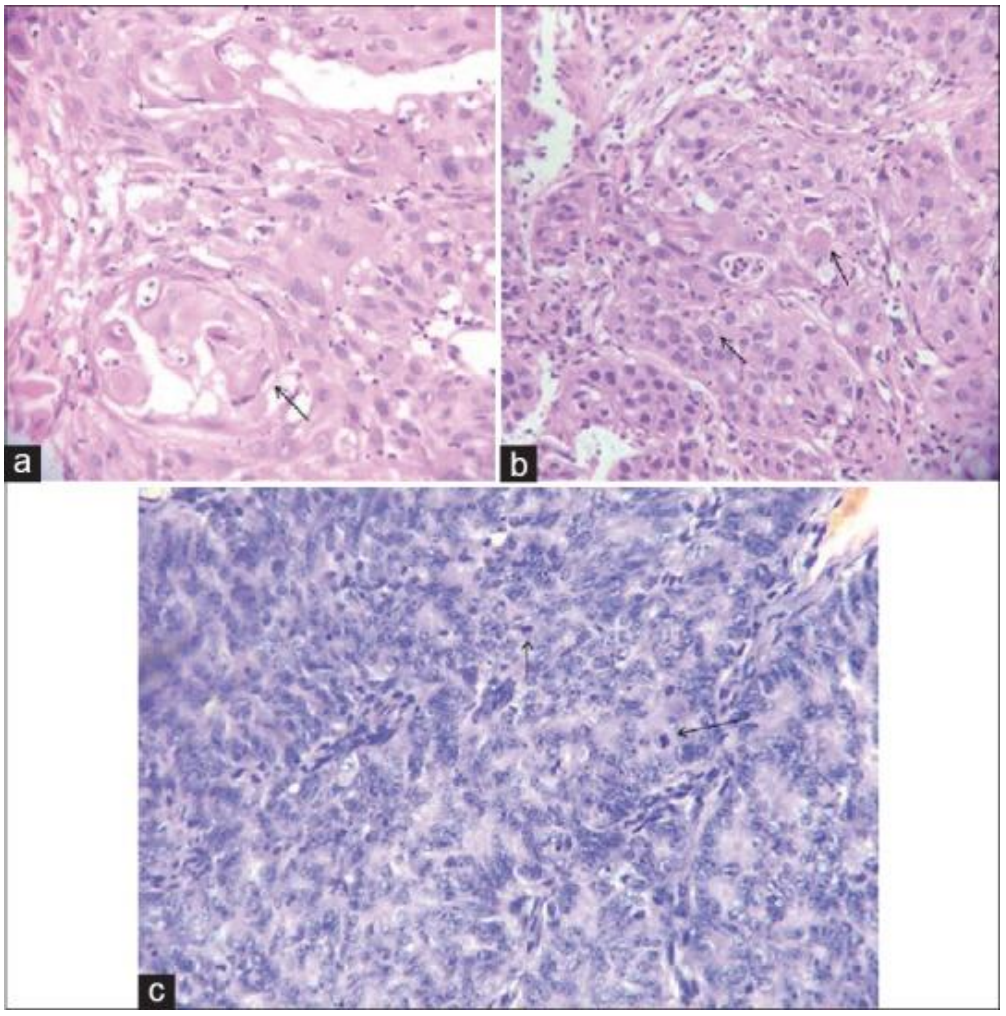


FIG 3: Cell differentiation. (a) ***Well-differentiated squamous cell carcinoma*** with mild pleomorphism, occasional mitotic figures and presence of keratin.

(b) ***Moderately differentiated squamous cell carcinoma*** with moderate nuclear pleomorphism, mitotic rate of 3/10 hpf and few keratin pearl formation

(c) ***Poorly differentiated squamous cell carcinoma*** with moderate to marked nuclear pleomorphism, absence of keratinization and increased mitosis >10/10 hpf

SALIVARY SAMPLE COLLECTION-

Subjects were instructed not to eat or drink for 30 minutes before the sample collection. They were seated comfortably on the dental chair in a well-ventilated area. The subjects were asked to rinse their mouths for 5 seconds with 10 ml distilled water. Following the spitting out of the water and initial swallow, whole saliva was collected without stimulation by spitting method directly into a sterile container for every 30 seconds for a duration of 2 minutes. If the collected salivary sample is insufficient, the collection was continued. From each subject approximately 2 ml of saliva was collected.

FIG 4: Saliva sample collection by spitting method

The collected saliva samples were labelled according to a system of letters and numbers, the letter indicated the group to which the patient belonged and the number indicating the order in which the samples were taken. The salivary samples were stored at a temperature of 20°C. The stored salivary samples were sent to the laboratory for estimation of sialic acid. The collected salivary samples were subjected to centrifugation at approximately 2000-3000 rpm for 20 min.

Salivary sialic acid levels were estimated by Enzyme- Linked Immune Sorbent assay (ELISA) method, using sialic acid kit. The normal sialic acid concentrations that were given as a guide line according to the kit were in the range of 0.2 mmol/l or 17.714 mg/dl.

KIT COMPONENTS:

- ✓ Standard solution (400mg/dl) – 0.5 ml
- ✓ Standard solution – 3 ml
- ✓ Coated ELISA plate – 12 well (8 tubes)
- ✓ Streptavidin -HRP – 6 ml
- ✓ Washing concentrate (30X)
- ✓ Anti SA antibodies labelled with biotin – 1 ml
- ✓ Stop solution – 6 ml

- ✓ Chromogen solution A – 6 ml
- ✓ Chromogen solution B – 6 ml
- ✓ Standard 135 ng/ml
- ✓ Seal plate membrane
- ✓ Hermetic bag

PRINCIPLE:

- This kit uses Enzyme-Linked Immune Sorbent assay (ELISA) based on the Biotin double antibody sandwich technology to assay the Human Sialic acid (SA).

- Add prepared samples and standards to the wells, which are pre-coated with Sialic acid (SA) monoclonal antibody and then incubate.

- After that, add anti SA antibodies labelled with biotin to unite with streptavidin-HRP, which forms immune complex.

- Remove unbound enzymes after incubation and washing.

- Add substrate A and B.

- Then the solution will turn blue and then into yellow with the effect of acid. The shades of solution and the concentration of Human Sialic acid (SA) are positively correlated.

ASSAY PROCEDURE:

1. Dilution of standard solutions:

200mg/di	Standard No.5	120µl Original Standard + 120µl Standard diluents
100mg/di	Standard No.4	120µl Standard No.5 + 120µl Standard diluents
50mg/di	Standard No.3	120µl Standard No.4 + 120µl Standard diluent
25mg/di	Standard No.2	120µl Standard No.3 + 120µl Standard diluent
12.5mg/di	Standard No.1	120µl Standard No.2 + 120µl Standard diluent

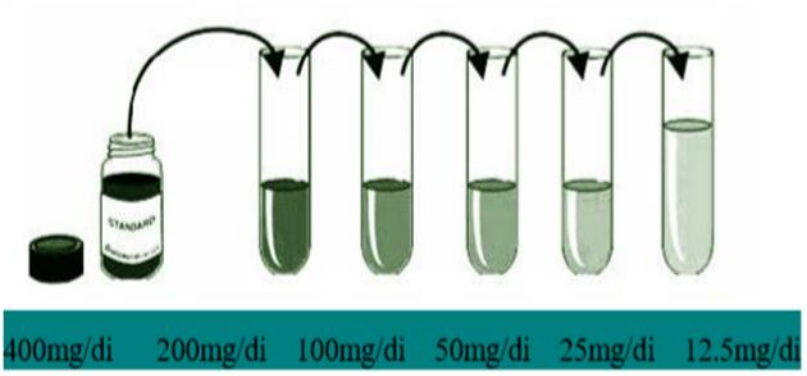


FIG 5: Dilution of standard solutions

The number of wells needed was determined by the number of samples to be tested added by that of standards. It was suggested that each standard solution

and each blank well should be arranged with three or more wells as much as possible.

2. Sample injection:

   - ✓ Blank well: no sample, anti SA antibody labelled with biotin or streptavidin-HRP is added to comparison blank well except chromogen solution A & B and stop solution while taking the same steps that follow.
   - ✓ Standard solution well: Add 50µl standard and streptomycin-HRP 50µl (biotin antibodies have united in advance in the standard so no biotin antibodies are added.)
   - ✓ Sample well to be tested: Add 40µl sample and then 10µl SA antibodies, 50µl streptavidin-HRP. Then cover it with seal plate membrane. Shake gently to mix them up. Incubate at 37°C for 60 minutes.

3. Preparation of washing solution: Dilute the washing concentration (30 X) with distilled water for later use.

4. Washing: Remove the seal plate membrane carefully, drain the liquid and shake off the remaining liquid. Fill each well with washing solution. Drain

the liquid after 30 seconds standing. Then repeat this procedure five times and blot the plate.

5. Colour development: Add 50μl chromogen solution A firstly to each well and then add 50μl chromogen solution B to each well as well. Shake gently to mix them up. Incubate for 10 minutes at 37℃ away from light for colour development.

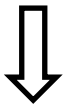
6. Stop: Add 50μl Stop Solution to each well to stop the reaction (the blue colour changes into yellow immediately at that moment).

7. Assay: Take blank well as zero, measure the absorbance (OD) of each well one by one under 450nm wavelength, which should be carried out within 10 minutes after having added the stop solution.

8. According to standards concentration and the corresponding OD values, calculate the linear regression equation of the standard curve. Then according to the OD value of samples, calculate the concentration of the corresponding sample.

SUMMARY:

Prepare reagents, samples and standards.



Add prepared samples and standards together with second antibody labelled with biotin and ELISA solutions. Let them react for 60 minutes at 37℃.

Wash the plate five times. Add Chromogen solution A and B. Incubate for 10 minutes at 37℃ for colour development.

Add stop solution

Read the OD value within 10minutes.

Calculate

Figures

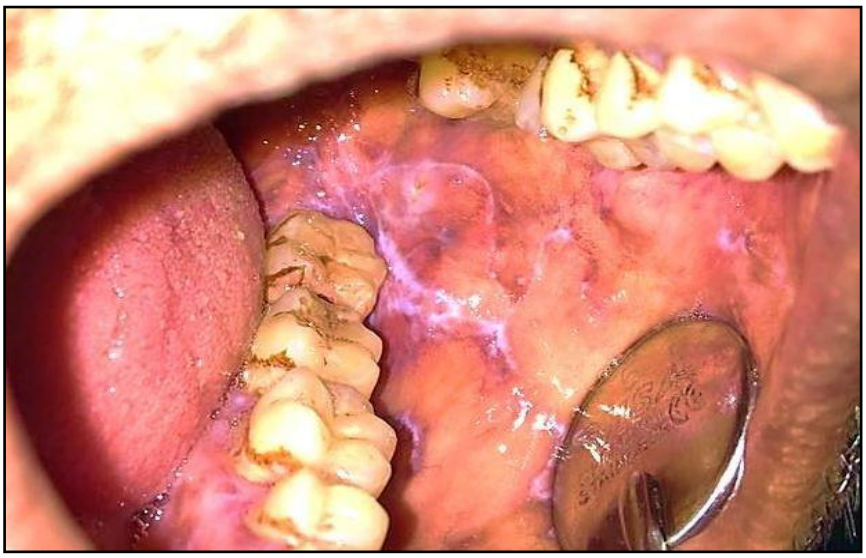


Fig 6: Lichen planus of left buccal mucosa

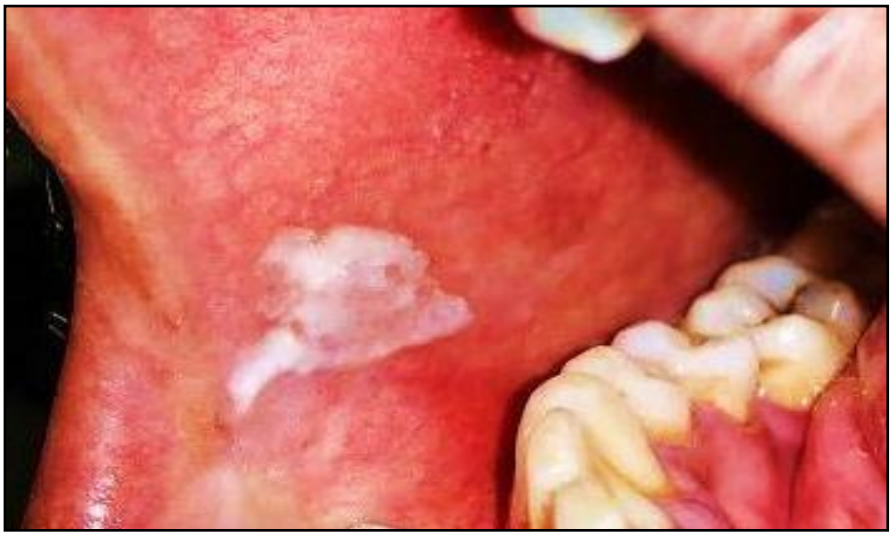


Fig 7: Leukoplakia of right buccal mucosa

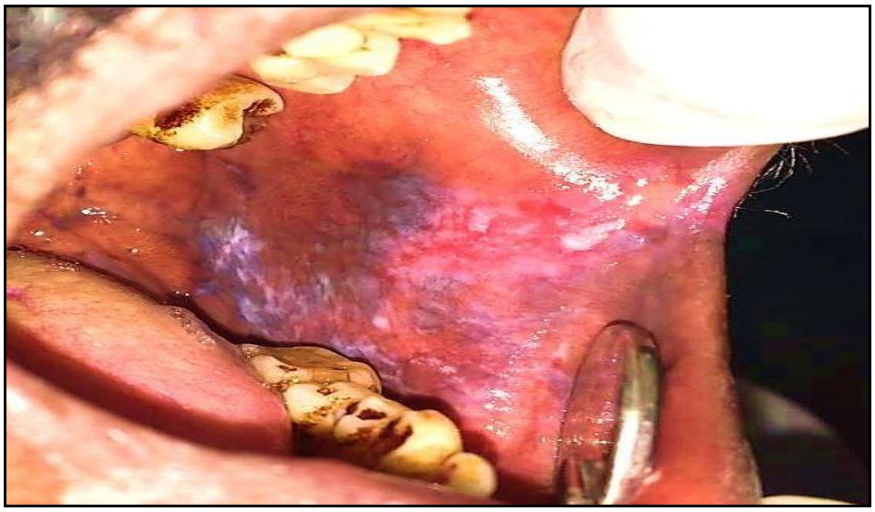


Fig 8: Speckled Leukoplakia of left buccal mucosa

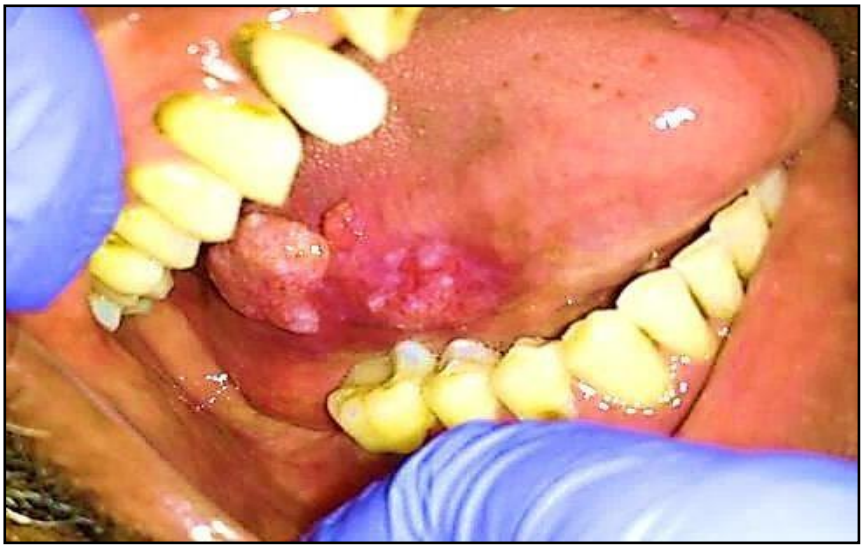


Fig 9: OSCC involving the tongue

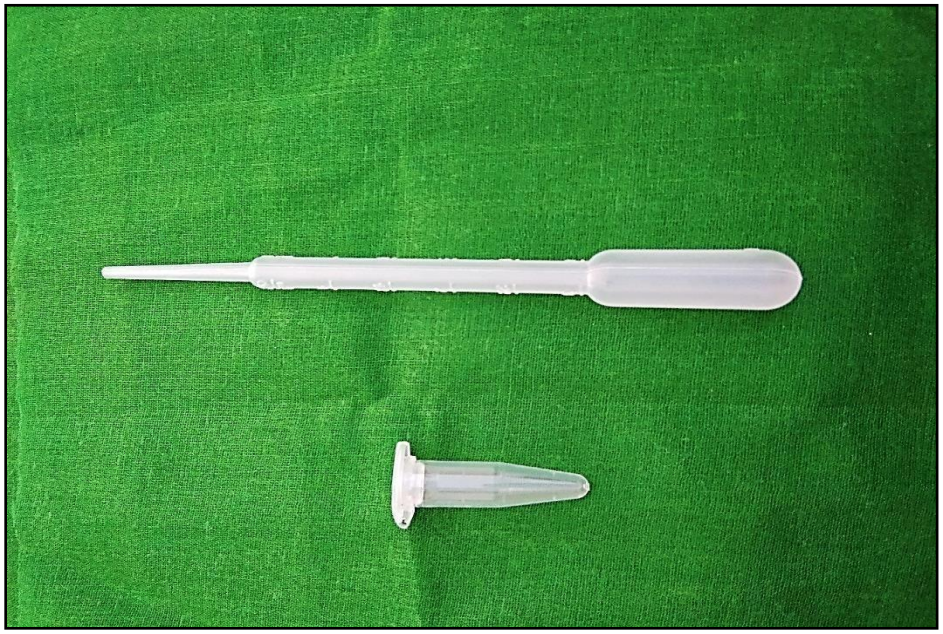


Fig 10: Pipette and centrifugation tube for saliva collection

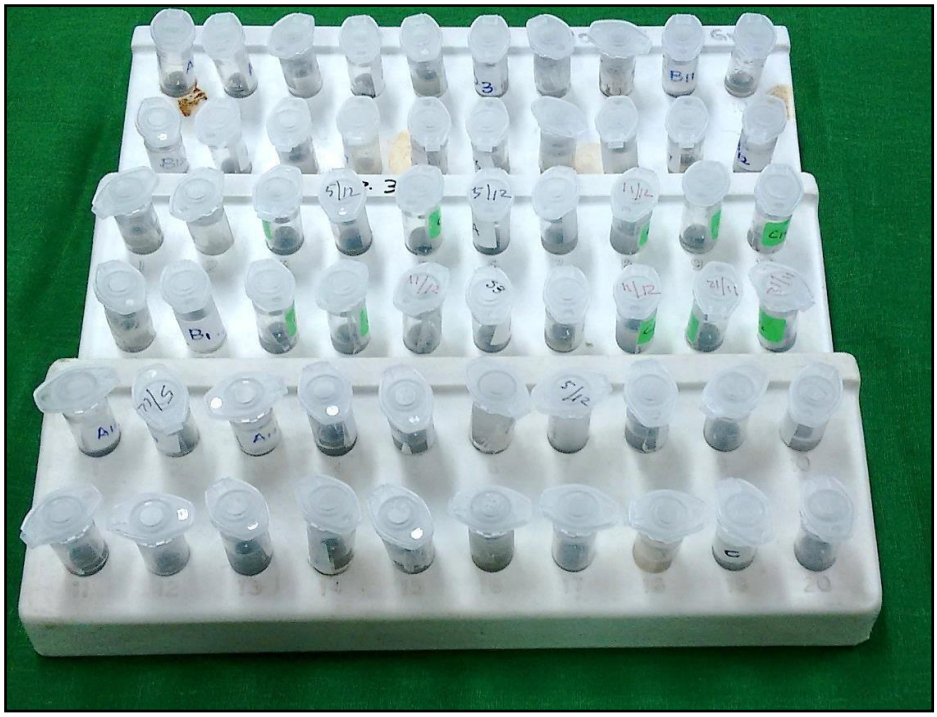


Fig 11: Salivary samples in the centrifugation tubes

Fig 12: Centrifugation of the sample

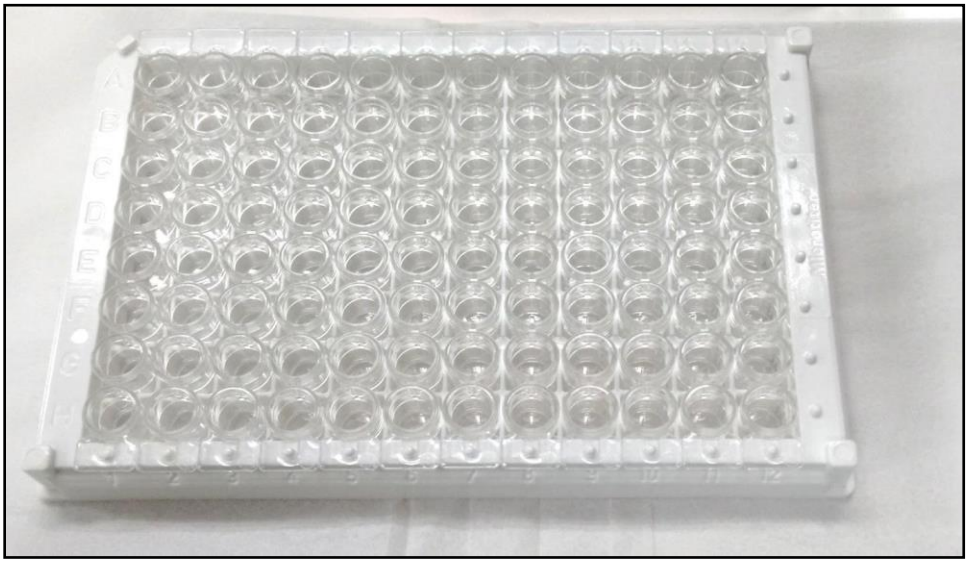


Fig 13: Microplate

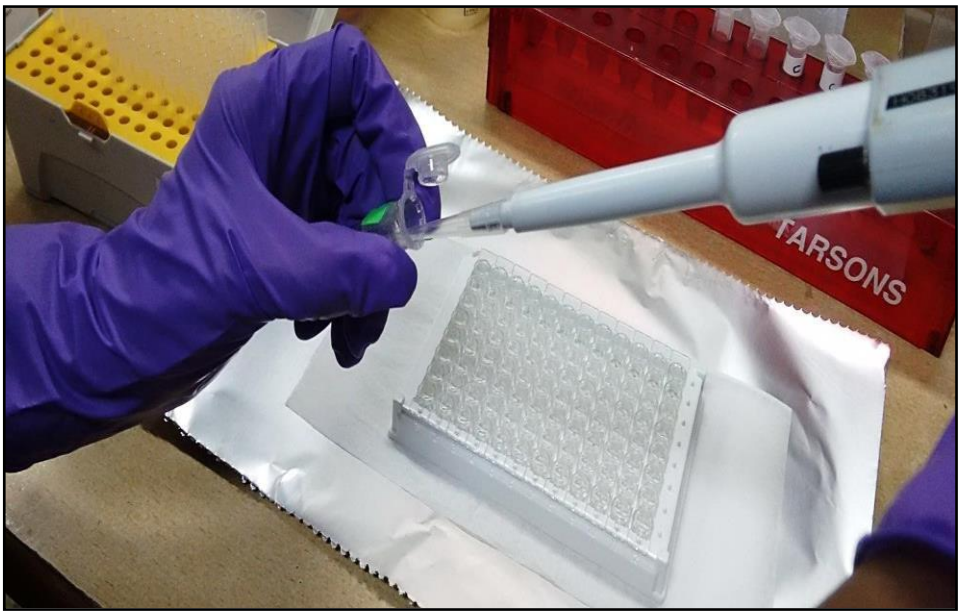


Fig 14: Adding of sample into the microplate

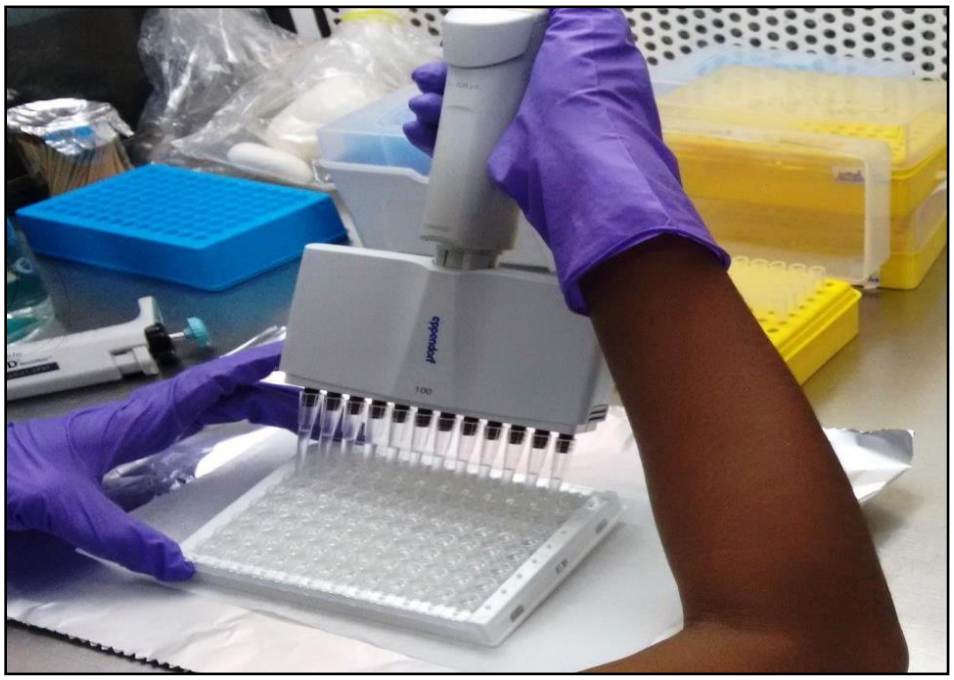


Fig 15: Washing procedure of each well

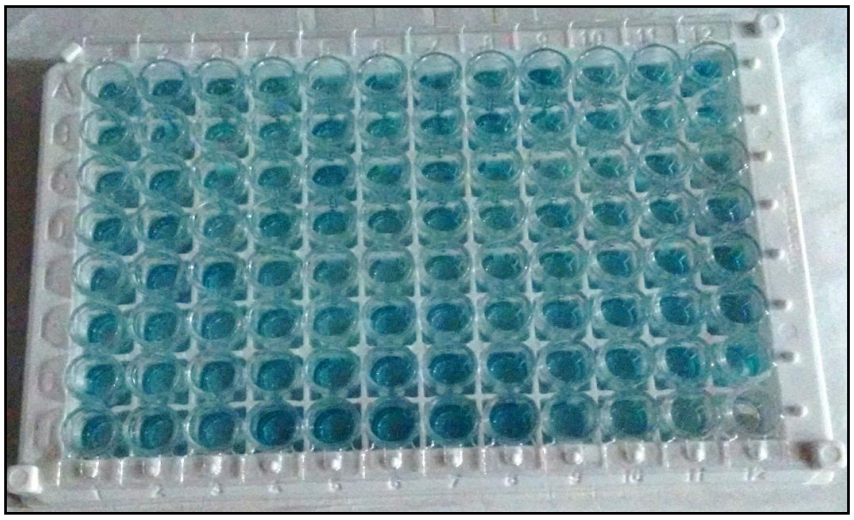


Fig 16: Change of colour (Blue) after adding substrate

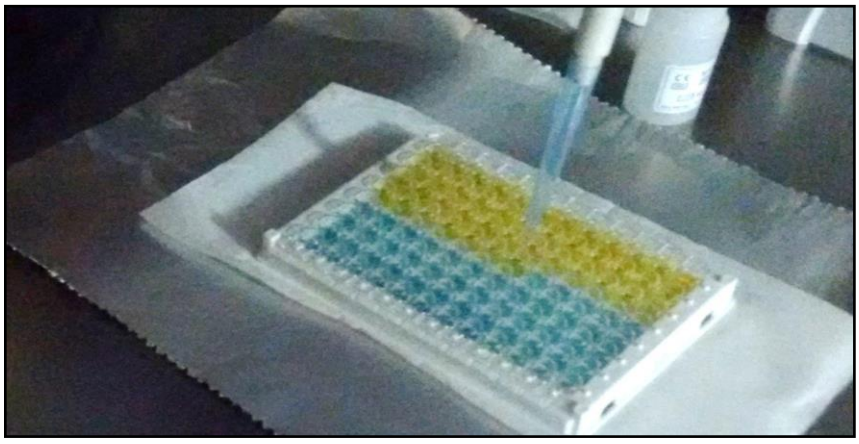


Fig 17: Appreciation of colour change (Yellow) after adding stop solution

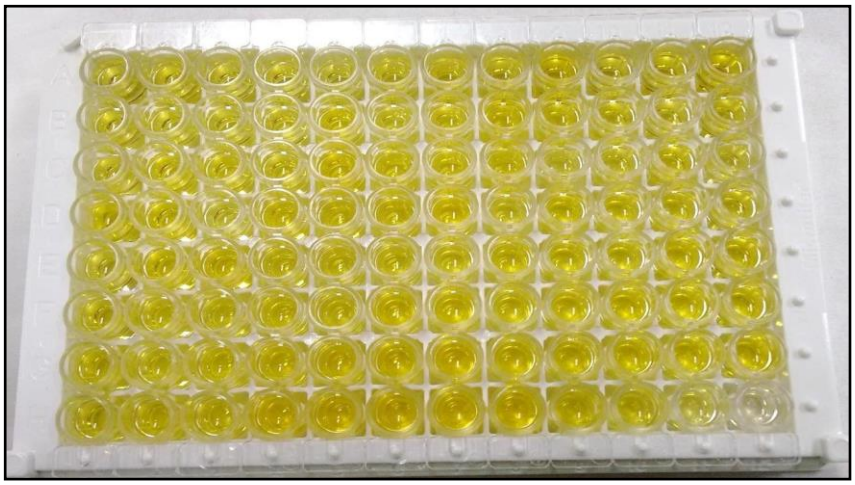


Fig 18: Colour intensity showing sialic acid concentration

Results

RESULTS

The present study is a randomized prospective case control study which was conducted in the Department of Oral medicine and Radiology of Ragas Dental College and Hospital, Uthandi, Chennai and Dr. Rai Memorial Cancer Institute, Teynampet, Chennai. The study was conducted between February 2019 to August 2019.

The study was devised to estimate and compare the salivary levels of sialic acid on a total of 60 subjects divided into three groups of 20 each oral squamous cell carcinoma patients, oral potentially malignant disorders and healthy controls and between various histopathological grades of OSCC.

The stored salivary samples were centrifugated and were analyzed in Aara laboratories, Roypettah, Chennai for the levels of salivary sialic acid with Enzyme Linked Immune Sorbent Assay (ELISA) method, using salivary sialic acid kit. The data obtained from the study were statistically analyzed. The results extracted were compared with various variables included in the study and are presented here.

Table 1 and graph I shows the mean salivary sialic acid levels in 20 OSCC patients (148.30 mg/dl), 20 oral PMD patients (70.70 mg/dl) and in 20 healthy controls (24.25 mg/dl) respectively.

Table 2 ang graph II shows group wise comparison of the salivary sialic acid levels by Post Hoc test , salivary sialic acid levels in Group I (OSCC) was greater than in group II (PMD) and group III (healthy controls) , the levels in group II (PMD) was lesser than in group I (OSCC) but greater than in group III (healthy controls) and the levels in group III (healthy controls) was lesser than in group I (OSCC) and group II (PMD). The p value obtained on group wise comparison of the study subjects was highly statistically significant with p value 0.000.

Table 3 and graph III shows the mean salivary sialic acid levels in OSCC patients with histopathological grading of WDSCC (105.17 mg/dl), MDSCC (131.86 mg/dl) and PDSCC (201.71 mg/dl) respectively.

Table 4 and graph IV shows group wise comparison of the salivary sialic acid levels in various histopathological gradings of OSCC by Post Hoc test, salivary sialic acid levels in WDSCC was lesser than in MDSCC and PDSCC, the levels in MDSCC was lesser than in PDSCC but greater than in WDSCC and the levels in PDSCC was greater than in WDSCC and MDSCC. The p value obtained on group wise comparison of the study subjects between WDSCC and PDSCC and between MDSCC and PDSCC was 0.000 which was highly significant. There was no statistically significant difference in the levels of salivary sialic acid between WDSCC and MDSCC with a p value of 0.098.

Tables and Graphs

TABLES AND GRAPHS

TABLE: 1 - MEAN SALIVARY SIALIC ACID LEVELS IN OSCC (GROUP I), PMD (GROUP II) AND HEALTHY CONTROLS (GROUP III)

SIALIC ACID LEVELS			
GROUPS	**N**	**RANGE (mg/dl)**	**MEAN ± (SD) (mg/dl)**
Group I	20	100-263	148.30 ± (46.483)
Group II	20	50-99	70.70 ± (12.541)
Group III	20	11-39	24.25 ± (7.725)

TABLE: 2- GROUP WISE COMPARISON BETWEEN OSCC (GROUP I), PMD (GROUP II) AND HEALTHY CONTROLS (GROUP III) BY POST HOC TEST

MULTIPLE COMPARISONS			
Dependent Variable: Sialic acid level **Tukey HSD**			
(I) Group	**(J) Group**	**Mean Difference (I-J)**	**P value**
Group I	Group II	77.600*	.000
	Group III	124.050*	.000
Group II	Group I	-77.600*	.000
	Group III	46.450*	.000
Group III	Group I	-124.050*	.000
	Group II	-46.450*	.000

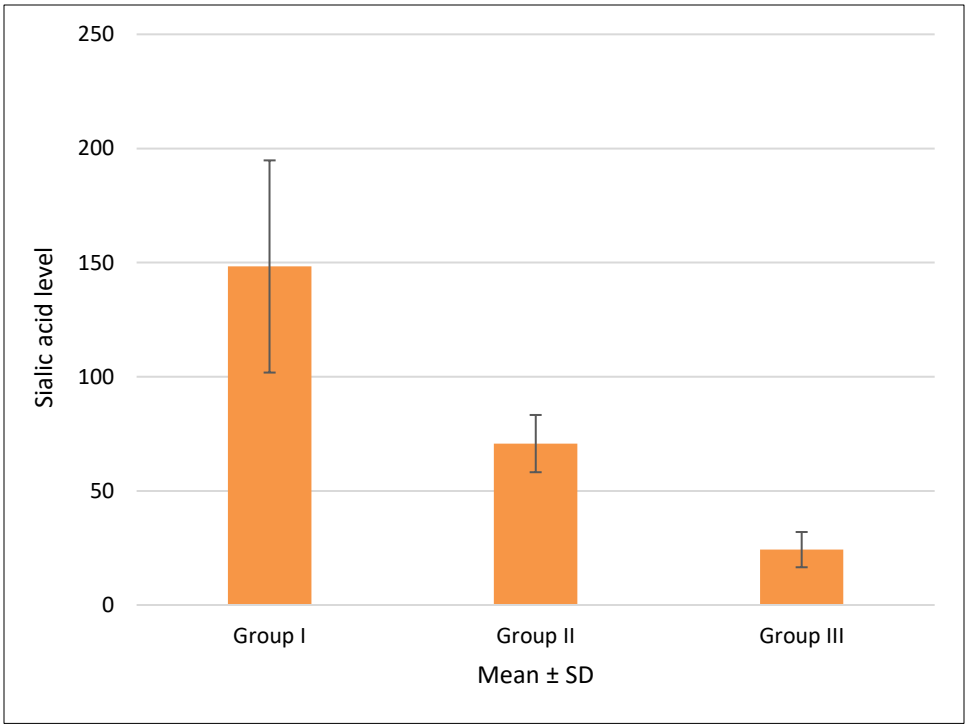
TABLE: 3 - MEAN SALIVARY SIALIC ACID LEVELS IN WDSCC, MDSCC AND PDSCC

OSCC			
HISTOPATHOLOGICAL GRADINGS	**N**	**RANGE (mg/dl)**	**MEAN ± (SD) (mg/dl)**
WDSCC	6	100 – 115	105.17 ± **(**6.585)
MDSCC	7	113 – 150	131.86 ± **(**13.656)
PDSCC	7	161 – 263	201.71 ± **(**33.390)

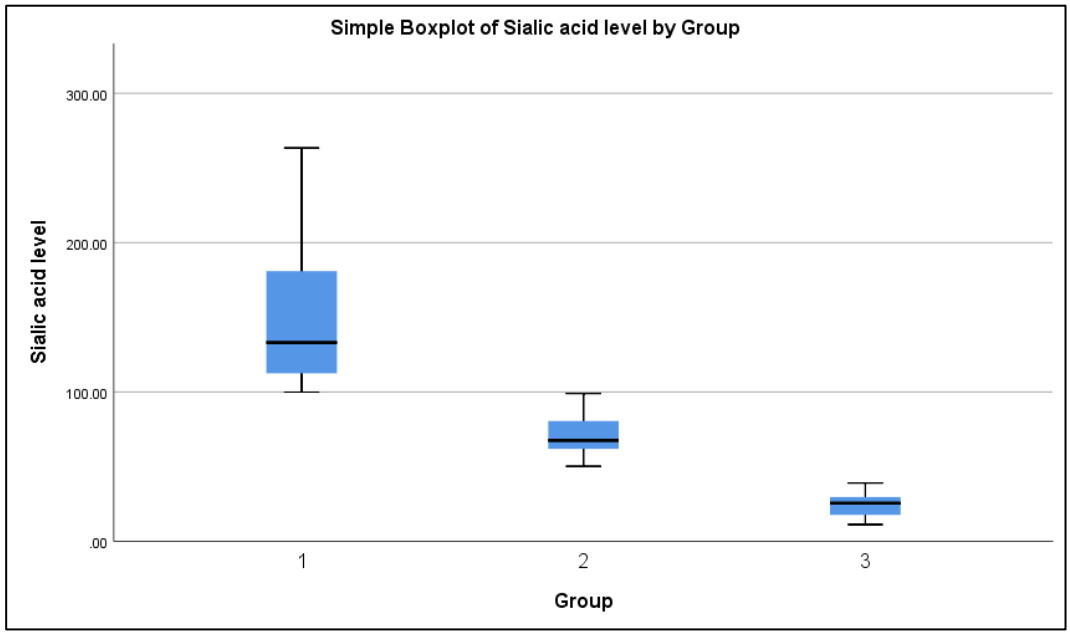
TABLE: 4 - GROUP WISE COMPARISON BETWEEN WDSCC, MDSCC AND PDSCC BY POST HOC TEST

(I) Histopathological grading	(J) Histopathological grading	Mean Difference (I-J)	P value
WDSCC	MDSCC	-26.690	.098
	PDSCC	-96.548*	.000
MDSCC	WDSCC	26.690	.098
	PDSCC	-69.857*	.000
PDSCC	WDSCC	96.548*	.000
	MDSCC	69.857*	.000
*. The mean difference is significant at the 0.05 level.			

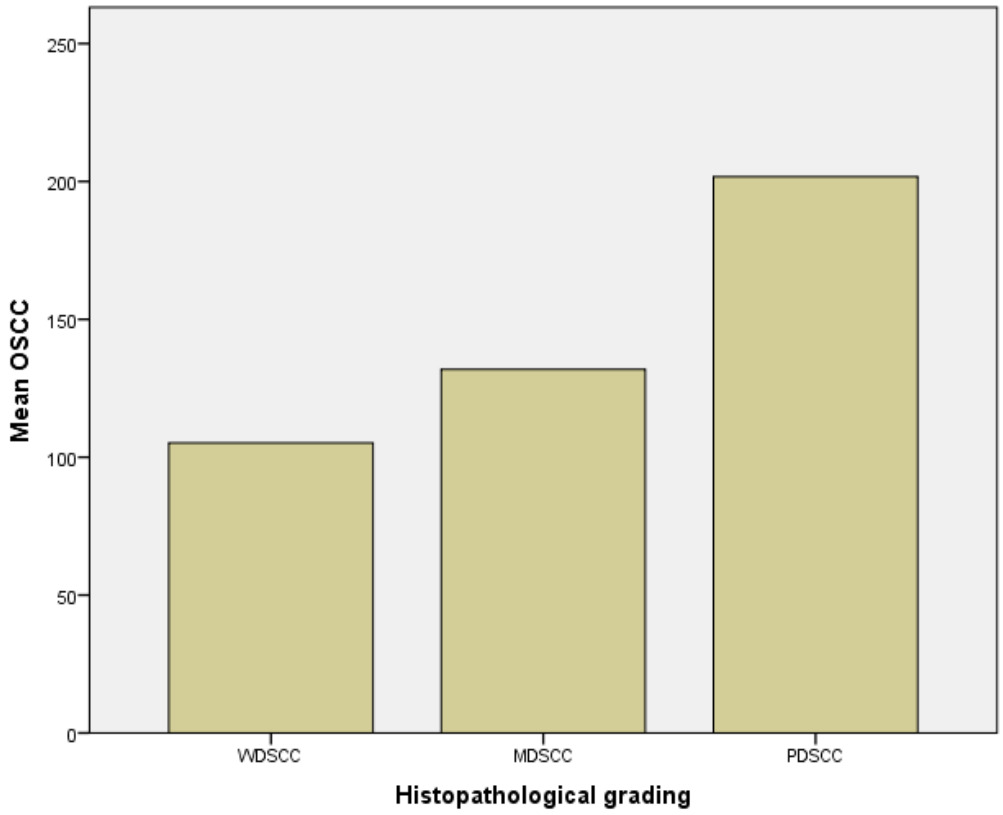
GRAPH I - MEAN SALIVARY SIALIC ACID LEVELS IN OSCC (GROUP I), PMD (GROUP II) AND HEALTHY CONTROLS (GROUP III)



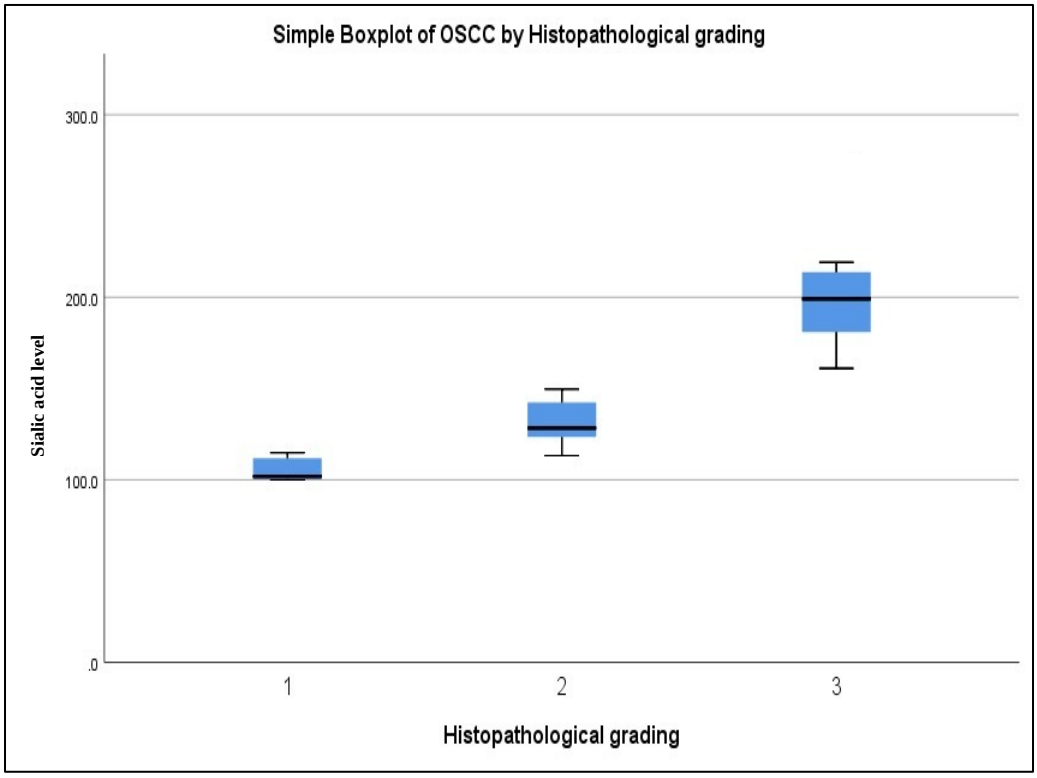
GRAPH II - GROUP WISE COMPARISON BETWEEN OSCC (GROUP I), PMD (GROUP II) AND HEALTHY CONTROLS (GROUP III)



GRAPH III - SHOWS MEAN SALIVARY SIALIC ACID LEVELS IN WDSCC, MDSCC AND PDSCC



GRAPH IV - GROUP WISE COMPARISON BETWEEN WDSCC, MDSCC AND PDSCC



Discussion

DISCUSSION

The cancer is one of the nemesis from the pre historical era that is affecting the morbidity and mortality of human life, but with time the effect of cancer has grown stronger due to the ill effects of various habits and other factors. Oral cancer is the sixth common cancer in humans worldwide with a high morbidity rate. India accounts for one-third of the world burden of oral cancer. Several studies had indicated the strong relation between tobacco and oral cancer. Many authors had suggested that the use of tobacco is the most common cause of oral cancer and since the use of tobacco is deeply embedded in Indian custom and culture, India is most targeted. The lack of public awareness, socio-economic status and easy accessibility contributed with the random use of tobacco related products. Tobacco products are available in both smokeless and smoked form and are being used by sizable number of people irrespective of age, sex and socio-economic status

Most of the oral cancers are preceded by potentially malignant disorders which generally have an increased risk of progression to cancer, but the risk varies depending upon a number of patient and lesion related factors. Therefore, it is a difficult task to predict the risk of progression in each and individual patient, and therefore the clinician must make a judgment only based on assessment of each

case. The most commonly encountered OPMDs are leukoplakia, oral submucous fibrosis, and erythroplakia [29].

The factors that are associated with an increased risk of malignant transformation include sex, site and type of lesion, habits such as tobacco usage in smoking and smokeless form, alcohol consumption and presence of epithelial dysplasia on histopathological examination [29]. It has been documented that about 5 – 18% of epithelial dysplasia are malignant.

In most cases oral cancer is diagnosed only when it becomes symptomatic, but by this stage most patients usually develop the advanced stages of the disease with regional metastasis and have consequently diminished prognosis.

So, early diagnosis of oral cancer is considered as the most important step than to treat the disease because it not only reduces the chance of spread of disease, but also rules out many invasive treatment strategies and increases the chances of normal survival

Many diagnostic aids are available for early diagnosis of oral cancer, but the role of biological markers is contested over others by various studies. Biological markers are used to diagnose cancer and to predict the therapeutic response and prognosis.

One such biological marker is sialic acid and in the past few decades the role of sialic acid as a tumor marker have been evaluated and advocated by various studies conducted in different parts of the world

The use of serum sialic acid as a tumor marker has been established in previous studies. However, its specificity is not yet determined. Saliva is basically an ultrafiltrate of blood and wins over blood as a diagnostic fluid because it is inexpensive, non-invasive and easy to handle. For patients, this non-invasive collection technique reduces discomfort and also simplifies procurement of repeated sampling for longitudinal monitoring over time [26]. Furthermore, the health-care experts prefer a salivary test than using serum because the latter is more likely to expose the technicians to various blood-borne diseases

Thus, our present study is to estimate and compare the salivary sialic acid levels in subjects with OSCC, PMD and healthy controls and between various histopathological grades of OSCC which serves as an effective tool for diagnosis and to predict the prognosis of malignancy as well as for monitoring post-treatment therapeutic response of the patients.

This is a prospective case control hospital-based study conducted between February 2019 to August 2019. The study was conducted among 60 subjects categorized into three groups. Group I consist of 20 patients with clinically and histologically proven oral squamous cell carcinoma, Group II consists of 20

patients with clinically and histologically proven PMD and group III consists of 20 healthy controls.

In the present study, the mean salivary sialic acid levels were found to be elevated in OSCC as compared to premalignant followed by the control group. The levels also showed statistically significant difference ($P < 0.05$) between the three groups.

The results of the present study are consistent with the results of the study done by **Sanjay et al [22] (2008), Chaudhari et al [26] (2016) and Achalli et al [15] (2017)**

Sanjay et al [22] (2008) in his study compared the levels of salivary sialic acid in control and malignant group and found that the levels are greater in malignant group when compared to that of control group. The study, however did not include a premalignant group.

Chaudhari et al [26] (2016) in his study, evaluated and correlated the salivary sialic acid levels among study group comprising of OSCC, PMD patients and control group comprising of healthy subjects and found that there was a highly significant difference between the two groups (i.e) the mean salivary sialic acid levels were found to be elevated in malignant as compared to premalignant followed by the control group.

Achalli et al [15] (2017) in his study also showed a progressive increase in salivary sialic acid levels from controls to PMD to OSCC.

In the present study, correlation of salivary sialic acid levels with various histopathological grading of OSCC was also done which shows that the mean salivary sialic acid level is elevated in grade III (poorly differentiated) followed by grade II (moderately differentiated) and grade I (well differentiated) and the levels were also statistically significant

The present study is consistent with study done by **Dharkar et al [23] (2013), Chaudhari et al [26] (2016) and Poudel et al [27] (2019)**

Dharkar et al [23] (2013) in his study found that there is increasing trends in salivary levels of sialic acid with increasing level of histopathological grades of OSCC.

Chaudhari et al [26] (2016) in his study also showed, that there is progressive increase in salivary sialic acid levels in various histopathological grades of OSCC (i.e) there is increase in salivary sialic acid levels in grade III (poorly differentiated) followed by grade II (moderately differentiated) and grade I (well differentiated).

Poudel et al [27] (2019) in his study also showed that there is significant increase in salivary sialic acid levels in PDSCC followed by MDSCC and WDSCC.

But, this result of the present study is not consistent with the study done by **Sanjay et al [22] (2008) and Shivashankar and Prabhu et al [30] (2011).**

Sanjay et al (2008) [22] found that salivary FSA levels were increased in WD cases as compared to MD cases. However, PBSA levels did not differ significantly among WD and MD cases. But, his discussion regarding study results were contradictory as he mentioned that there is correlation of elevated salivary sialic acid levels to the progression of OSCC. But, **Chaudhari et al (2016) [26]** has cited Sanjay et al (2008) which is contradictory, because in his study there is elevated sialic acid levels in PDSCC followed by MDSCC and WDSCC which is not consistent with the present study.

Similarly, **Shivashankar and Prabhu et al [30] (2011) in his study** also found increased levels of FSA and PBSA in WD cases as compared to MD cases. More studies with a greater number of specimens are required to give an explanation to this finding.

The possible reason for such variation in the results could be subjective variation between histopathologic grading and the grading system used. Furthermore, in the present study, seven Grade III cases were studied. If, we consider the clinical staging of these histopathologically diagnosed Grade III cases, all seven cases belonged to stage IV. Hence, tumor burden and lesser degree of differentiation might be the causes of higher levels of sialic acid in Grade III cases.

Summary and Conclusion

SUMMARY AND CONCLUSION

The present study titled ***"Estimation of salivary sialic acid in oral premalignancy and oral squamous cell carcinoma"*** was conducted in the Department of Oral medicine and Radiology, Ragas Dental College, Uthandi, Chennai to estimate salivary levels of sialic acid in study group comprising of patients with OSCC and oral potentially malignant disorders and to compare the same with control group comprising of healthy subjects and between various histopathological grades of OSCC.

A total of 60 subjects were included in the study. Among the 60 subjects, 20 each of OSCC and PMD were included in the study group and 20 healthy subjects were included in the control group. Salivary samples were collected from the study group and control group and sialic acid levels were estimated using sialic acid ELISA kit.

The results of the present study were documented as follows –

- Salivary sialic acid levels were found to be elevated in OSCC followed by oral precancer and healthy subjects
- Salivary sialic acid levels were elevated in PDSCC followed by MDSCC and WDSCC.

The results of the present study infer that salivary sialic acid may be used as a tumor marker for early diagnosis of oral cancer as well as to predict its prognosis since the procedure is simple and non-invasive.

Very few studies have been done on salivary sialic acid estimation and its use as a diagnostic tool would go a long way in early diagnosis of oral cancer. However, a longitudinal study with a larger sample size is needed to evaluate the authenticity of these parameters as specific tumor markers.

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Annexures

ANNEXURE -I

CASE SHEET

RAGAS DENTAL COLLEGE & HOSPITAL

2/102, EAST COAST ROAD, UTHANDI, CHENNAI – 600119

DEPARTMENT OF ORAL MEDICINE & RADIOLOGY

ESTIMATION OF SALIVARY SIALIC ACID IN ORAL PREMALIGNANCY AND ORAL SQUAMOUS CELL CARCINOMA

Date:

S.No :

OP.No :

Patient's name:

Age/Sex:

Address:

Phone number:

Occupation:

Monthly income:

Past medical /surgical/dental /history:

Personal history:

ETIOLOGY	**PRESENT**	**ABSENT**
Smoking		
Tobacco chewing		
Sharp tooth		
Others		

Provisional Diagnosis:

Investigation & Reports:

Final Diagnosis:

Study group: Group I / Group II / Group III

ANNEXURE –II

RAGAS DENTAL COLLEGE AND HOSPITAL

DEPARTMENT OF ORAL MEDICINE AND RADIOLOGY

CONSENT LETTER

I ....., the undersigned hereby give my consent for the performance of salivary study "ESTIMATION OF SALIVARY SIALIC ACID IN ORAL PREMALIGNANCY AND ORAL SQUAMOUS CELL CARCINOMA" by S. EZHIL PALLAVI under the able guidance of Dr S. KAILASAM, M.D.S., Professor and Head of the Department, Department of Oral Medicine and Radiology, Ragas Dental College and Hospital, Chennai-600119. I have been informed and explained the procedure and the purpose of the study. I also understand and accept this as a part of the study protocol there by voluntarily, unconditionally and freely give my consent without any fear or pressure in a mentally sound and conscious state to participate in the study.

Witness/Representative: Patient's signature:

Date:

RAGAS DENTAL COLLEGE AND HOSPITAL

DEPARTMENT OF ORAL MEDICINE AND RADIOLOGY

ஒப்புதல் கடிதம்

நான் _____என்னுடைய முழு ஒத்துழைப்பை சு. எழில் பல்லவி அவர்கள் மற்றும், திரு. மரு. சு. கைலாசம், பேராசிரியர் மற்றும் முதன்மை மருத்துவர், வாய் மருத்துவம் மற்றும் வாய்நோய் அறிதல் கதிர் வீச்சுத்துறை, ராகாஸ் பல் மருத்துவ முதுநிலை படிப்பிற்கான அவர்களுடைய "**உமிழ்நீரில் உள்ள சியாலிக் அமிலம் அளவினை வாய்புற்றுநோய் தன்மை உடைய சிதைவு மற்றும் வாய்புற்றுநோயினில் கண்டறிதல்**" குறித்த ஆய்வில் நான் என் முழு சுயநினைவில் யாருடைய வற்புறுத்தலும் இல்லாமல், யாருடைய கட்டுப்பாட்டிற்க்கு கீழ் பணியாமலும் என்னுடைய ஒத்துழைப்பை இந்த மருத்துவ ஆராய்ச்சிக்காக ஒப்புதலையும் அளிக்கின்றேன் .

சாட்சிகள்: **கையொப்பம்**

தேதி:

ANNEXURE –III

RAGAS DENTAL COLLEGE & HOSPITAL
(Unit of Ragas Educational Society)
Recognized by the Dental Council of India, New Delhi
Affiliated to The Tamilnadu Dr. M.G.R. Medical University, Chennai - 600 032
2/102, East Coast Road, Uthandi, Chennai - 600 119. INDIA
Tele : (044) 2453 0002 - 06. Principal (Dir) 2453 0001 Fax : (044) 24530009

TO WHOM SO EVER IT MAY CONCERN

Date: 20.12.2019

Place: Chennai

From

The Institutional Review Board

Ragas Dental College and Hospital

Uthandi, Chennai – 119

The Project titled **"ESTIMATION OF SALIVARY SIALIC ACID IN ORAL PREMALIGNANCY AND ORAL SQUAMOUS CELL CARCINOMA"** submitted by Dr. **S. Ezhil Pallavi** has been approved by the Institutional Review Board of Ragas Dental College and Hospital.

Dr. N.S .Azhagarasan, MDS
Member secretary,
The Institutional Review Board
Ragas Dental College and Hospital
Uthandi, Chennai – 119

RAGAS DENTAL COLLEGE Chennai

ANNEXURE –IV

URKUND

Urkund Analysis Result

Analysed Document:	Estimation of salivary sialic acid in oral premalignancy and oral sqaumous cell carcinoma.pdf (D62530071)
Submitted:	1/17/2020 11:24:00 AM
Submitted By:	${Xml.Encode(Model.Document.Submitter.Email)}
Significance:	7 %

Sources included in the report:

dissertation final document.doc (D34112125)
ASSESSMENT OF SALIVARY CORTISOL LEVELS AMONG PATIENTS WITH ORAL SQUAMOUS CELL CARCINOMA, POTENTIALLY MALIGNANT DISORDERS AND HEALTHY CONTROLS.docx (D35005896)
print (2).pdf (D61791127)
CORRELATION OF SERUM LEVO - FUCOSE LEVELS AMONG PATIENTS WITH ORAL SQUAMOUS CELL CARCINOMA, ORAL POTENTIALLY MALIGNANT DISORDERS AND HEALTHY CONTROLS.docx (D35078833)
https://www.researchgate.net/publication/216701942_Evaluation_of_salivary_sialic_acid_total_protein_and_total_sugar_in_oral_cancer_A_preliminary_report
https://www.ncbi.nlm.nih.gov/pubmed/28836530
https://www.researchgate.net/publication/49646685_Estimation_and_comparative_study_of_serum_total_sialic_acid_levels_as_tumor_markers_in_oral_cancer_and_precancer
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6503791/
https://www.ncbi.nlm.nih.gov/pubmed/31110436
https://rd.springer.com/article/10.1007/s10719-018-9820-0
https://www.researchgate.net/scientific-contributions/69025733_Z_S_Wang
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3385277/
https://www.researchgate.net/publication/319188450_Sialic_acid_as_a_biomarker_of_oral_potentially_malignant_disorders_and_oral_cancer
https://www.researchgate.net/publication/328954888_Unique_Journal_of_Medical_and_Dental_Sciences_SERUM_AND_SALIVARY_SIALIC_ACID_AND_L-FUCOSE_AS_PROGNOSTIC_MARKERS_IN_POTENTIALLY_MALIGNANT_DISORDERS_AND_ORAL_CANCER
https://www.researchgate.net/publication/5900413_Warnakulasuriya_S_Johnson_NW_van_der_Waal_INomenclature_and_class

ANNEXURE –V

SAMPLE NO.	NS (mg/dl)	PMD (mg/dl)	OSCC (mg/dl)
1	39	61.91	101.6
2	33.6	99	114.8
3	15.3	81.6	111.8
4	17.1	82.6	101.9
5	11.2	78.2	99.9
6	19	56.8	100.4
7	25.1	68.9	137.9
8	28.1	79.5	125.6
9	35	62	146.8
10	31.3	67	121.3
11	29.4	67.3	128.3
12	17.1	88.3	149.7
13	15.3	81.7	113.2
14	18.9	67.8	186.9
15	18.3	66.1	208.4
16	29.3	50.3	161
17	27.1	60.2	263.4
18	30	52.3	219.2
19	21.3	76.1	174.8
20	26.2	66	199.1

SAMPLE NO.	OSCC (mg/dl)	Histopathological grading
1	101.6	1
2	114.8	1
3	111.8	1
4	101.9	1
5	99.9	1
6	100.4	1
7	137.9	2
8	125.6	2
9	146.8	2
10	121.3	2
11	128.3	2
12	149.7	2
13	113.2	2
14	186.9	3
15	208.4	3
16	161	3
17	263.4	3
18	219.2	3
19	174.8	3
20	199.1	3