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***Research Article***

# CHANGING BIOCHEMICAL MARKERS AND ONGOING PROCESS OF TRANSFORMATION…A PILOT STUDY

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## ABSTRACT

The role of oxygen free radicals in the initiation, promotion and progression of carcinogenesis and the protective role of anti-oxidant defenses has been the subject of much speculation in the recent past with conflicting reports in the literature. In recent years, increasing experimental and clinical data have provided compelling evidence for the involvement of oxidative stress in a large number of pathological states including cancers. Purpose of Study was to measure the concentration of serum total proteins and albumin as potent anti-oxidants and advanced oxidation protein products (AOPP) as markers of oxidant mediated protein damage in sera of patients diagnosed with speckled leukoplakia, one of the most common oral pre-cancerous lesions with high malignant transformation rates and well-differentiated oral squamous cell carcinoma. The study consisted of sera analysis of total protein, albumin and AOPP levels in patients with speckled leukoplakia and histologically proven well-differentiated oral squamous cell carcinoma. One way Analyses of Variance (Anova) was used to test the difference between groups. To find out which of the two groups means was significantly different, post hoc test of Scheffe was used.
Results: The study revealed variations in sera levels of albumin and advanced oxidation protein products to be statistically significant. The results obtained emphasize the need for more studies with larger sample sizes to be conducted before a conclusive role for sera levels of total protein, albumin and AOPP could be drawn as markers of transition from the various oral pre-cancerous lesions and conditions to frank oral squamous cell carcinoma. A study highlighting the possibility of using serum total protein, albumin and AOPP levels as possible, reliable markers in the early detection of the changes contributing towards the transformation of the various oral pre-malignant lesions and conditions into frank oral squamous cell carcinoma.

***Key Words:*** *Reactive Oxygen Species, Carcinogenesis, Free Radicals, Antioxidants, Transformation, Pre-Cancerous*

## INTRODUCTION

Oral squamous cell carcinoma is one of the most common malignant neoplasms worldwide and is the most common cancer in the males and the third most common cancer in the females in India. In India, about 60,000 new cases of oral cancer are reported to occur every year with tobacco consumption being the single most important risk factor for the development of oral cancers. Bursts of reactive oxygen species in tobacco users have long been implicated as the prime form of damage brought to the genetic material leading to non-lethal mutations eventually turning out in the form of frank malignant lesions in this group of individuals. (Kolanjiappan *et al.,* 2003)

Oral cancer has a much higher prevalence in the elderly age group with this higher prevalence among the elderly population explained on the basis of an age related increase in the magnitude of the attack of the oral carcinogens as free radicals including the so-called reactive oxygen and nitrogen species, [ROS and RNS] causing various DNA mutations and aberrations. It may also result from an age related reduction in

the body's defense mechanisms including the body's antioxidant defenses. (Hershkovich *et al.,* 2007; and Khanna *et al*., 2005)
Also, the development of cancer is multi-factorial depending on the extent of damage brought to the DNA which, in turn, is proportional to the magnitude of reactive oxygen and nitrogen stresses. This is only when this equilibrium is disturbed that the damage to the DNA is brought about and cancer evolves. (Kolanjiappan *et al*., 2003; Bahar *et al.,* 2007; and Erol *et al*., 2007)
In plasma, free thiol groups are quantitatively the most important scavengers of the various free radicals and are known to be located largely on the various serum proteins, one amongst them being albumin. Advanced oxidation protein products, formed as a result of irreparable oxidative damage to the proteins, have been, on the other hand, defined as novel and reliable markers of the irreversible oxidative damage.
Despite tremendous advances in the diagnosis and the management of oral cancers, this group of cancers is considered to be the one with the highest mortality as well morbidity rates with the diagnostic adjuncts which are used to aid an early diagnosis of oral cancers either suffer from a lack of sensitivity in the initial stages of the processes leading to frank oral cancers or suffer from a setback of not being so cost effective.
In addition, biopsy, which is considered to be the gold standard in the diagnosis of oral cancers, suffers from the reliability of an appropriate site for the obtainment of the specimen to be conclusive. The introduction of the concept of the field of cancerization has further questioned the significance of biopsy results in the approval or, rejection of the reports that come out to be confirmative of either dysplastic or, frank cancerous changes seen in the tissue.
The role of biochemical markers, on the other hand, comes out to be a convincing enough evidence of the changes taking place in the body eventually turning out to develop into frank malignant degenerations. The alteration of serum chemistry and the outpouring of the various growth factors and cytokines and tumor markers in the early enough changes leading to frank oral cancers is an added boon in the early diagnosis at a time when tissue and cell level changes are not obvious to be taken as an evidence in this regard.
The role of serum total protein and albumin as plasma's potent anti-oxidant defenses and advanced oxidation protein products as markers of oxidant mediated protein damage, if comes out to be a convincing enough evidence to be used as reliable markers of oxidative stress in the body, could be helpful in the early identification and even more significantly, in determining the pre-disposition of the various oral pre-cancerous lesions and conditions, into their transformation to frank oral cancers.
Hence, the present study is being planned to assess the levels of serum total protein, albumin and advanced oxidation protein products in normal, healthy individuals and the individuals afflicted with speckled leukoplakia, one of the most common oral pre-cancerous lesions with high malignant transformation rates, against its transformation into frank oral squamous cell carcinoma.

## MATERIALS AND METHODS

**Source of Data:** The study was conducted in the Dept. of Oral Medicine and Radiology, Govt. Dental College and Research Institute, Bangalore for a period of 3 months from Jan 2010 to March 2010. The study consisted of 30 new cases of clinically diagnosed and histologically proven well-differentiated, oral squamous cell carcinoma, 10 patients with speckled leukoplakia aged between 40-60 years in addition to 25 healthy controls.
**Method of collection of Data:** None of the patients were on any therapeutic modality prior to the inclusion in the study or, suffering from any systemic condition, especially hepatic or, renal disorders with or, without dialysis that could have affected serum total protein, albumin as well as AOPP levels. The sera of the subjects were obtained taking full precautions to prevent hemolysis. Bio-chemical analysis of serum total protein, albumin and advanced oxidation protein products was done in the Dept. of Clinical Biochemistry, Bangalore Medical College and Research Institute and associated Hospitals, Bangalore.

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Assessment of serum total protein, albumin and advanced oxidation protein products: Bio-chemical analysis of serum total protein, albumin and advanced oxidation protein products was done in the Dept. of Clinical Biochemistry, Bangalore Medical College and Research Institute and associated Hospitals, Bangalore.

**Collection of blood and serum separation:** For this, following an overnight fasting period, 5 ml of venous blood was taken from selected patients from the antecubital vein using a sterile disposable syringe in the sitting position between 8 A.M. and 10 A.M. Serum was immediately separated by ultracentrifugation taking full precautions to prevent hemolysis. The supernatant was discarded and the rest of the sample was stored at -20 degrees Celsius.

Assay of serum total protein and albumin in sera - Serum levels of total protein and albumin was done with the help of Biuret method. (Khanna *et al.,* 2005; and Bahar *et al.,* 2007) Serum total protein and albumin were expressed as g/dL.

**Biuret test**: The biuret test is a chemical test used for detecting the presence of peptide bonds. In the presence of peptides, a copper (II) ion forms a violet-colored complex in an alkaline solution. Several variants on the test have been developed.

The Biuret reaction can be used to assay the concentration of proteins because peptide bonds occur with the same frequency per amino acid in the peptide. The intensity of the color, and hence the absorption at 540 nm, is directly proportional to the protein concentration, according to the Beer-Lambert law. (Khanna *et al.,* 2005; and Bahar *et al.,* 2007)

Assay of advanced oxidation protein products in sera - Advanced oxidation protein products were measured by spectrophotometry. The assay was calibrated using chloramine-T and the absorbance was read at 340 nm on a microplate reader. Advanced oxidation protein products' concentration was expressed as micromol/L of chloramine-T equivalents. (Erol *et al.,* 2007; and Yasunori *et al,* 2006)

Spectrophotometry: Spectrophotometry is the quantitative measurement of the reflection or transmission properties of a material as a function of wavelength. Spectrophotometry involves the use of a spectrophotometer. A spectrophotometer is a photometer (a device for measuring light intensity) that can measure intensity as a function of the light source wavelength. Important features of spectrophotometers are spectral bandwidth and linear range of absorption or reflectance measurement. (Erol *et al.,* 2007; and Yasunori *et al,* 2006)

**Method of Statistical Analysis:**

The following methods of statistical analysis have been used in this study:

The results were averaged (mean +/- standard deviation) for continuous data and number and percentage for dichotomous data have been presented in Tables and Figures.

One way Analysis of Variance (Anova) was used to test the difference between groups.

To find out which of the two groups means was significantly different, post hoc test of Scheffe was used.

For comparison of two variances, $S_a^2$ and $S_b^2$, estimated for two groups, $N_a$ and $N_b$ subjects respectively, F test was used wherein:

$$F = \frac{Sa^2}{Sb^2}$$

with $N_a$-1 and $N_b$-1 degrees of freedom.

In above test, P values less than 0.05 were taken to be statistically significant.

The data was analysed using SPSS (version 10.5).

The normality of data was checked using **Kolmogorov-Smirnov** and **Shapiro-Wilk** tests for significance before the statistical analysis was performed [table attached for reference, Table.1].

**Table 1: Table depicting tests of normality of data**

| | Group | Kolmogorov-Smirnov | | | Shapiro-Wilk | | |
|---|---|---|---|---|---|---|---|
| | | Statistic | df | Sig. | Statistic | df | Sig. |
| **Serum Total Protein** | **Control** | 0.133 | 25 | 0.200 | 0.961 | 25 | 0.427 |
| | **Speckled leukoplakia** | 0.327 | 10 | 0.003 | 0.744 | 10 | 0.003 |
| | **Well-differentiated, Oral Squamous cell carcinoma** | 0.123 | 30 | 0.200 | 0.932 | 30 | 0.055 |
| **Serum Albumin** | **Control** | 0.092 | 25 | 0.200 | 0.969 | 25 | 0.611 |
| | **Speckled leukoplakia** | 0.139 | 10 | 0.200 | 0.962 | 10 | 0.804 |
| | **Well-differentiated, Oral Squamous cell carcinoma** | 0.109 | 30 | 0.200 | 0.953 | 30 | 0.198 |
| **Serum Advanced Oxidation Protein Products** | **Control** | 0.166 | 25 | 0.075 | 0.901 | 25 | 0.019 |
| | **Speckled leukoplakia** | 0.188 | 10 | 0.200 | 0.930 | 10 | 0.447 |
| | **Well-differentiated, Oral Squamous cell carcinoma** | 0.147 | 30 | 0.098 | 0.841 | 30 | 0.000 |

**RESULTS**

While the mean values of serum total protein were much the same in controls (8.236 +/- 1.5025 g/dL) as against the cases diagnosed with speckled leukoplakia (9.85 +/- 3.6788 g/dL) and well-differentiated, oral squamous cell carcinoma (7.8 +/- 3.1500 g/dL) [Table.2], there were observed great variations in the minimum (1.6 g/dL) to the maximum values (18.2 g/dL) for well-differentiated, oral squamous cell carcinoma. The p value however came out to be statistically insignificant implying the role of various other factors in protein metabolism in cancer patients [Table.2].

Serum albumin levels however came out to be statistically significant ($p<0.001$) [Table.3] with serum albumin levels as low as 1.7 g/dL in frank oral squamous cell carcinoma as against a minimum of 3 g/dL in the control group. The mean values of serum albumin came out to be 4.956 +/- 1.0579 g/dL in the control group [Table.3]

**Table 2: Table depicting mean serum total protein in study groups along with standard deviation and P values**

| | Serum Total Protein (g/dL) | | | | | | 'p' value |
|---|---|---|---|---|---|---|---|
| | Control [n=25] | | Speckled leukoplakia [n=10] | | Well-differentiated, Oral Squamous cell carcinoma [n=30] | | |
| | Mean | SD | Mean | SD | Mean | SD | |
| | 8.236 | 1.5025 | 9.85 | 3.6788 | 7.8 | 3.1500 | 0.1305 |
| **P1** | - | | 0.295 | | 0.841 | | |
| **P2** | - | | - | | 0.130 | | |

*P1: Comparison between Control, Speckled leukoplakia and Well-differentiated, oral squamous cell carcinoma groups*
*P2: Comparison between Speckled leukoplakia and Well-differentiated, oral squamous cell carcinoma groups*

**Table 3: Table depicting mean serum albumin in study groups along with standard deviation and P values**

| | Serum Albumin(g/dL) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control [n=25] | | Speckled leukoplakia [n=10] | | Well-differentiated, Oral Squamous cell carcinoma [n=30] | | 'p' value |
| | Mean | SD | Mean | SD | Mean | SD | |
| | 4.956 | 1.0579 | 3.79 | 0.9410 | 3.6933 | 1.2177 | <0.001 |
| **P1** | - | | 0.026* | | <0.001* | | |
| **P2** | - | | - | | 0.972 | | |

*P1: Comparison between Control, Speckled leukoplakia and Well- differentiated, oral squamous cell carcinoma groups*
*P2: Comparison between Speckled leukoplakia and Well- differentiated, oral squamous cell carcinoma groups*
**p<0.05*

Advanced oxidation protein products, also, revealed marked variations in controls and the patients diagnosed with frank squamous cell carcinomas (0.42563 +/- 0.2010 micromol/L) with p value<0.001 [Table.4]. The mean value of serum advanced oxidation protein products was found to be 0.0788 +/- 0.0279 micromol/L in the control group [Table.4]. Sera levels of advanced oxidation protein products shot-up to 0.918 micromol/L in the frank oral squamous cell carcinoma group as against a minimum of 0.041 micromol/ L in the control group [Table.4]. The mean value of serum advanced oxidation protein products in patients diagnosed with speckled leukoplakia also came out to be significantly high (0.368 +/- 0.0978 micromol/L) [Table.4].

**Table 4: Table depicting mean serum advanced oxidation protein products in study groups along with standard deviation and P values**

| | Serum Advanced Oxidation Protein Products (micromol/L) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control [n=25] | | Speckled leukoplakia [n=10] | | Well-differentiated, Oral Squamous cell carcinoma [n=30] | | 'p' value |
| | Mean | SD | Mean | SD | Mean | SD | |
| | 0.0788 | 0.0279 | 0.368 | 0.0978 | 0.42563 | 0.2010 | <0.001 |
| **P1** | - | | <0.001* | | <0.001* | | |
| **P2** | - | | - | | 0.549 | | |

*P1: Comparison between Control, Speckled leukoplakia and Well- differentiated, oral squamous cell carcinoma groups*
*P2: Comparison between Speckled leukoplakia and Well- differentiated, oral squamous cell carcinoma groups*
**p<0.05*

**Table 5: Table depicting comparison of serum total protein, albumin and advanced oxidation protein products in study groups along with the mean difference, standard error and significance**

| Dependent Variable | (I) Group | (J) Group | Mean Difference (I-J) | Std. Error | Sig. |
|---|---|---|---|---|---|
| **Total Protein (g/dL)** | **Control** | **Speckled leukoplakia** | -1.61400 | 1.02331 | 0.295 |
| | **Control** | **Well-differentiated, Oral Squamous cell carcinoma** | 0.43600 | 0.74062 | 0.841 |
| | **Speckled leukoplakia** | **Well-differentiated, Oral Squamous cell carcinoma** | 2.05000 | 0.99865 | 0.130 |
| | | | | | |
| **Albumin (g/dL)** | **Control** | **Speckled leukoplakia** | 1.16600 | 0.41922 | 0.026 |
| | **Control** | **Well-differentiated, Oral Squamous cell carcinoma** | 1.26267 | 0.30341 | <0.001 |
| | **Speckled leukoplakia** | **Well-differentiated, Oral Squamous cell carcinoma** | 0.09667 | 0.40911 | 0.972 |
| **Advanced Oxidation Protein Products (micromol/L)** | **Control** | **Speckled leukoplakia** | -0.289200 | 0.053677 | <0.001 |
| | **Control** | **Well-differentiated, Oral Squamous cell carcinoma** | -0.346833 | 0.038848 | <0.001 |
| | **Speckled leukoplakia** | **Well-differentiated, Oral Squamous cell carcinoma** | -0.057633 | 0.052383 | 0.549 |

The level of significance came out to be less than 0.001 in case of sera levels of albumin in between the controls and well-differentiated, oral squamous cell carcinoma patients [Table.5]. The study also revealed statistically significant results in between the controls and patients diagnosed with speckled leukoplakia

and controls and the patients afflicted with well-differentiated, squamous cell carcinomas being less than 0.001 [Table.5].
The results arrived at confirmed the results obtained from other studies in relation to sera levels of total protein and albumin while again were in concordance with the results obtained in the published studies in relation to advanced oxidation protein products in relation to general body cancers. The statistically insignificant results obtained in the patients diagnosed with speckled leukoplakia and oral squamous cell carcinoma groups were explained on the basis of multiple, confounding factors that play a significant role in protein metabolism in cancer patients.

**DISCUSSION**
Oxidative stress is a general term used to describe the steady state level of oxidative damage in a cell, tissue or, organ, caused by the reactive oxygen species .This damage can affect a specific molecule or, the organism as a whole. (Yasunori *et al.,* 2006) Reactive oxygen species such as free radicals and peroxides represent a class of molecules that are derived from the metabolism of oxygen and exist inherently in all aerobic organisms. Most reactive oxygen species are generated from the endogenous sources as byproducts of normal and essential metabolic reactions such as energy generation from mitochondria or, the detoxification reactions involving the hepatic microsomal enzyme system. Exogenous sources include exposure to cigarette smoke, environmental pollutants such as emission from the automobiles and industries, consumption of alcohol in excess, asbestos, and exposure to ionizing radiation in addition to the plethora of the bacterial, fungal and viral infections. (Bahar *et al.,* 2007)
The determinants of oxidative stress are regulated by an individual's unique hereditary factors as well as environment and characteristic lifestyle. Unfortunately, under the present day life style conditions, many people run an abnormally high level of oxidative stress that could increase their probability of early incidence of decline in optimum body functions and lead to a number of pathologies. (Elango *et al.,* 2006) Most free radicals are highly reactive and short lived. (Kolanjiappan *et al.,* 2005) Sun has proposed that free radicals are involved in both the initiation and the promotion of multistage carcinogenesis. These free radicals have been shown to cause DNA damage, activate pro-carcinogens and alter the cellular anti-oxidant defense mechanisms. (Khanna *et al.,* 2005)
Plasma is known to contain a wide range of important antioxidants including albumin, ascorbic acid and uric acid. In contrast, concentrations of enzymes such as super-oxide dismutase, reduced glutathione and catalase, all of which are known to be important intracellular antioxidants, are low in plasma. While ascorbate is an important extra-cellular antioxidant, albumin via its thiol groups, provides quantitatively almost ten folds greater antioxidant protection against the various reactive oxygen and nitrogen species held responsible for the genetic damage eventually leading to the development of cancers. (Kolanjiappan *et al.,* 2003; Bahar *et al.,* 2007; and Elango *et al.,* 2006)
The analysis of changes in serum total protein in malignancy is in itself a means of studying abnormality in the protein metabolism in this condition. Until recently, radical induced damage to proteins was considered to be mainly a chain-terminating process. It was thought that the products of damage produced on the protein, as a result of protein scission, cross-linking, chemical modification of side chains, were relatively inert with the intermediaries subsequently degraded by intra-and extra-cellular enzymes. It has recently been demonstrated however that these intermediaries are capable of initiating further chemical reactions thereby leading to the depletion of important cellular reductants such as ascorbates and glutathione via redox reactions. (Erol *et al.,* 2007; Yasunori *et al.,* 2006; and Ihara *et al.,* 2004) Serum total protein in our study came out to be statistically insignificant implying the role of the several complex factors that may play a role in protein metabolism in cancer patients as held by the numerous other studies conducted earlier in this regard. (Hans *et al.,* 2006)
In humans, albumin is the most abundant plasma protein accounting for about 55-60% of the measured serum proteins. It consists of a single polypeptide chain of 585 amino acids with a molecular weight of

around 66,500 Da. The mature, circulating molecule is arranged in a series of alpha-helices, folded and held by 17 disulphide bridges. (Nicholson *et al.,* 2000)

Albumin synthesis takes place only in the liver and secreted into the portal circulation as soon as it is formed. The rate of synthesis varies with nutritional and disease states. (Hans *et al.,* 2006; Nicholson et al., 2000; and Hans *et al*., 1996) Amongst the numerous plasma proteins that possess anti-oxidant properties owing to their rich concentrations of free thiol groups, albumin is unusual in having a free sulfhydryl group in addition. (Nicholson *et al.,* 2000)

With normal concentrations lying between 3.5-5.5 g/dL, the serum level of albumin is related mainly to its synthesis and catabolism. In fact, only a small number of factors are known to result in variation in serum albumin. In addition, it has been reported that serum albumin decreases with age and cigarette smoking with the usual half-life of albumin being 20 days. (Nicholson *et al.,* 2000; Ferdinand *et al*., 1999; and James and Hay, 1968)

Several lines of evidence suggest strongly that a reduced serum albumin concentration, although within the normal range, is associated with increased mortality risk. (Nicholson *et al.,* 2000; and Akinori *et al.*, 2004) From studies performed with healthy subjects and patients, it has been reported that the estimated increase in the odds of death ranges from 24 to 56 % for each 2.5 g/L decrement in serum albumin concentration. The serum albumin level thus appears to be an independent predictor of mortality risk with a direct protective effect of the albumin molecule being suggested by the persistence of the association after adjustment for other risk factors. Albumin may thus represent quantitatively the most important component that plays a determinant role in the efficient antioxidant defense, organisms have developed to protect against oxidative attack. (Nicholson *et al.,* 2000; Kouoh *et al.,* 1999; James and Hay, 1968; Soejima *et al*., 2004; and Jonathan and Ellen, 2001)

Albumin in our study came out to be statistically significant with values varying from a minimum of 2 g/dL to 5.1 g/dL in patients diagnosed with speckled leukoplakia to as low as 1.7 g/dL in patients afflicted with frank oral squamous cell carcinoma. This is in concordance with the observations of the several studies conducted in the past that laid emphasis on the protective role of albumin as one of the most abundant extracellular antioxidant available in the plasma of patients diagnosed with frank squamous cell carcinomas. The exact role of albumin in assessing the prognosis is, therefore, warranted by larger, follow-up studies correlating the level of serum albumin in these groups of patients with the overall 5-year survival rates.

Also, cells can generally remove oxidized proteins by proteolysis. However, certain oxidized proteins are poorly handled by cells and this may contribute to the observed accumulation and damaging actions of oxidized proteins during aging and various other pathologies, even cancers. (Hans *et al.,* 2006; Nicholson et al., 2000; and Hans *et al*., 1996)

Advanced oxidant protein products, first described by Witko-Sarsat et al. (1996), further have been hypothesized to activate the endothelial cells and to a lesser extent, fibroblasts to generate reactive oxygen species. (Erol *et al.,* 2007) Furthermore, advanced oxidation protein products generated by different oxidation patterns lead to the production of either NO or, $H_2O_2$ suggesting their role in the generation of different types of reactive oxygen species that set a cascade of reactions with a potential to damage cellular micro-molecules eventually turning out into frank oral squamous cell carcinoma. (Erol *et al.,* 2007; and Servettaz *et al*, 2007).

The level of advanced oxidation protein products in our study ranged from a minimum 0.242 micromol/L to 0.534 micromol/L in patients diagnosed with speckled leukoplakia to as high as 0.918 micromol/L in patients diagnosed with histologically proven well-differentiated, oral squamous cell carcinoma. The results obtained however could not be compared with the observations of other studies as the AOPP levels have been assessed in relation to other cancers of the body in the studies conducted in the past. This study deserves the credit of being the first of its kind assessing the sera levels of AOPP as one of the important diagnostic marker in patients diagnosed with oral cancers.

**Conclusion**

Reactive oxygen and nitrogen stresses have long been implicated in the genesis of oral cancers. There is enough literature available that shows convincing evidence in the use of anti-oxidants as chemo-preventive agents to halt the transformation of various oral pre-cancerous lesions and conditions into frank oral cancers. The results obtained emphasize the need for more studies to be conducted in this regard for the assessment of sera levels of total protein, albumin and advanced oxidation protein products to accept their utility and to assess their role in the pathogenesis and their impact on the prognosis of oral cancers providing a scientific ground for the use of diverse chemo-preventive strategies in controlling damage at genetic and molecular levels to prevent the ongoing transition of various oral pre-cancerous lesions and conditions into frank malignant degenerations .

Contributions from the authors: Literature search, manuscript preparation, manuscript editing and manuscript review.

Ethical Declaration: The study has been approved by the ethical committee appointed by the Government Dental College and Research Institute, Bangalore and Bangalore Medical College and Research Institute, Bangalore and has therefore been performed in accordance with the ethical standards laid down in the 1964 declaration of Helsinki and its later amendments after a written informed consent from the patients for their inclusion in the study. Details that might disclose the identity of the patient have been omitted.

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