Research Article

iMedPub Journals
www.imedpub.com

Annals of Clinical and Laboratory Research
ISSN 2386-5180

2020
Vol.8 No.2:312

DOI: 10.36648/2386-5180.8.2.312

Biochemical Test of Serum Ceruloplasmin as a Biomarker for Early Detection of Oral Potentially Malignant Epithelial Lesions (PMELs) and Oral Squamous Cell Carcinoma (OSCC)

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Citation: Chandran A, Nachiappan S, Tripuraneni SC, Das M, Manikandan G, Kothakapu KR, Nayyar AS (2020) Biochemical Test of Serum Ceruloplasmin as a Biomarker for Early Detection of Oral Potentially Malignant Epithelial Lesions (PMELs) and Oral Squamous Cell Carcinoma (OSCC). Ann Clin Lab Res. Vol.8 No.2:312.

Abstract

Context and Aim: Despite the recent advances in cancer treatments, the outcome and prognosis of the various oral cancers is still relatively poor. The lacuna for this lies in the delayed and late diagnosis of neoplasms when the tumor is already in advanced stages of the disease. An early enough diagnosis is, thus, highly warranted to initiate treatment in the initial stage itself to arrest the progression of the malignant process. Such measures, therefore, are of great help and desired fundamental for an early detection of this dreaded disease so as to ensue an early treatment. The purpose of the present study is to estimate and evaluate the efficacy of serum ceruloplasmin levels as a potential biomarker in the early detection of oral potentially malignant epithelial lesions (PMELs) and frank oral cancers.

Materials and Methods: The present observational study was conducted over a period of 2 years wherein a total of 100 subjects aged between 18 to 60 years clinically diagnosed and histopathologically confirmed with oral leukoplakia, oral submucous fibrosis (OSMF) and oral squamous cell carcinoma (OSCC) between the age range of 20-60 years were included. The patients were subjected to incisional biopsy after routine hematological investigation. The same sera samples were, then, used for the analysis of serum ceruloplasmin levels.

Statistical Analysis Used: The statistical analysis was carried-out using the Statistical Package for Social Sciences (SPSS version 17.0, EPI-INFO 6.0 version). Comparison of serum ceruloplasmin levels with the control group was performed using one way Analysis of Variance (one way ANOVA) test (F-Test) while frequencies were compared with the help of chi-square test. Inter-group comparisons and multiple comparisons were done with the help of Tukey's Test. $P < 0.05$ was considered statistically significant.

Results: On comparing serum ceruloplasmin levels in the patients of the four groups, statistically significant difference was found in all the four groups ($p < 0.05$).

Conclusion: The observations of the present study revealed that serum ceruloplasmin levels were found to be raised in all the three groups including oral leukoplakia, OSMF and OSCC patients as compared to the controls and thus, can be used as a potential biomarker in the early detection of oral potentially malignant epithelial lesions (PMELs) and frank oral cancers.

Key words: Serum Ceruloplasmin, Biomarker, Early Detection, Oral Potentially Malignant Epithelial Lesions (PMELs), Oral Squamous Cell Carcinoma

Received: June 14, 2019, **Accepted:** April 07, 2020, **Published:** April 14, 2020

Introduction

Industrialization and urbanization have raised the standards of life and concomitantly, increased the levels of stress in the modern era. The modern man, unable to cope this stress, finds alternative ways to relieve this stress and falls prey to the addictions of gutkha chewing, tobacco, pan, smoking and alcohol etc. These habits, though bring transient euphoria in the addicted individuals, are harmful in the long run, invariably, leading to potentially malignant lesions like oral leukoplakia, erythroplakia and oral submucous fibrosis (OSMF), eventually, turning into obvious oral cancers. Oral potentially malignant epithelial lesions (PMELs) are defined as those lesions and/or, conditions of the oral mucosa that are dysplastic but not frankly malignant but are more prone to turning into frank malignant degenerations [1]. The term pre-cancerous lesion/condition has been discarded since not all these lesions/conditions turn into malignancies. Thus, these changes of the mucosa are referred to as potentially malignant epithelial lesions (PMELs) [2,3].

Oral cancer is the most common malignancy known in the head and neck region and is one of the major causes of deaths worldwide. Approximately 80,000 new cases of oral cancers are diagnosed each year, mainly, due to consumption of different forms of available tobacco products such as gutkha, quid, snuff or, misri [4]. Annually, around one laks and thirty thousand people succumb to oral cancers which translates into approximately 14 deaths per hour in India [5]. The most commonly encountered oral neoplasm is oral squamous cell carcinoma (OSCC) and it accounts for 95 % of all the oral cancers reported [6]. Squamous cell carcinoma has been defined by Pindborg JJ and Sirsat SM [7] as a malignant epithelial neoplasm exhibiting squamous differentiation characterized by the formation of keratin and/or, the presence of intercellular bridges. All potentially malignant epithelial lesions (PMELs), eventually, progress to develop invasive OSCCs [6].

Despite the recent advances in cancer treatments, the outcome and prognosis of OSCC is still poor. The lacuna for this lies in the delayed and late diagnosis of neoplasm when the tumor is already in advanced stages of the disease [8]. An early enough diagnosis is, thus, highly warranted to initiate treatment in the initial stage itself to arrest the progression of the malignant process. Such measures are of great help and desired for an early detection of this dreaded disease process so as to ensue an early treatment, thereby, reducing the mortality and morbidity, in case the patient survives, up to a certain level. Tumor markers are specific substances released either by the tumor or, the host while combating the tumor into the serum. The identification of such substances, thus, helps in the prediction of disease progression and is, thus, of utmost significance in the management of these lesions [9]. Altered concentration of these biomarkers in the serum or, saliva of an individual, then, gives the signal of the future alarming condition pertaining to the process of frank malignant transformations [10].

Oxidative stress is an imbalance resulting out of free radical damage and mechanism of antioxidant protection in the body. Cupric ions are reported to inhibit the production of singlet oxygen and this is of particular significance because of its ability to cross the cell membrane and its high reactivity towards various biomolecules [11]. Oxidative stress might lead to compensatory increased levels of the serum ceruloplasmin levels in patients with such imbalances. Greater than normal ceruloplasmin levels are noticed in numerous cancers including the leukemias, Hodgkin's lymphomas [12,13]. The purpose of the present study was to estimate and evaluate the efficacy of serum ceruloplasmin levels as a potential biomarker in the early detection of oral potentially malignant epithelial lesions (PMELs) including oral leukoplakia and OSMF and frank OSCC patients.

Materials and Methods

The present observational study was conducted over a period of 2 years wherein a total of 100 subjects aged between 18 to 60 years clinically diagnosed and histopathologically confirmed with oral leukoplakia, OSMF and OSCC between the age range of 20-60 years were divided into four groups with each group consisting of 25 patients as Group A consisting of 25 healthy controls who had no major illness in the recent past, Group B with 25 patients clinically diagnosed with oral leukoplakia, Group C with 25 patients clinically diagnosed with OSMF and Group D with 25 patients clinically diagnosed with OSCC. Patients with present or, past history of any major illness such as liver disease, diabetes, hypertension and tuberculosis, patients undergoing radiotherapy or, chemotherapy for cancer, patients with a history of malignancy other than oral cancers and individuals > 60 years of age with severely compromised immunity were excluded. Biopsy was considered as the gold standard for the confirmation of the said diagnoses. Ethical clearance was obtained from the Institutional Ethics Committee while the subjects were informed in detail regarding the need and protocol of the study and a written, informed consent was obtained from them before their inclusion in the study. The patients were, then, examined thoroughly and a detailed case history was recorded in a specially designed proforma. The patients were subjected to incisional biopsy taking tissue from the periphery of the lesions along with adjacent normal tissue after routine hematological investigation. The same sera samples were used for the analysis of serum ceruloplasmin levels with the help of Human Ceruloplasmin Enzyme-Linked Immuno-sorbent Assay kit (Assay Max) manufactured by CUSABIO Assay Max. The estimation of serum ceruloplasmin levels was done using Houchin O Boyd [14] method which was a colorimetric assay.

Statistical Analysis Used: The statistical analysis was carried-out using the Statistical Package for Social Sciences (SPSS version 17.0, EPI-INFO 6.0 version). Comparison of serum ceruloplasmin levels with the control group was performed using one way Analysis of Variance (one way ANOVA) test (F-Test) while frequencies were compared with the help of chi-square test. Inter-group comparisons and multiple comparisons were done with the help of Tukey's Test. $P < 0.05$ was considered statistically significant.

Results: **Table 1** reveals age-wise distribution of the patients showing that 64 % of the patients in group A, 8 % in group B and

36 % in group C were in the age group of 20-29 years while 32 % in group A, 24 % in group B, 20 % in group C and 4 % in group D were in the age group of 30-39 years. As the age advanced, 4 % of the patients in group A, 52 % in group B, 28 % in group C and 24 % in group D were in the age group of 40-49 years and 16$% in group B and C and 64 % in group D were in the age group of 50-59 years respectively. By using chi-square test, statistically significant difference was found in the mean ages of the patients in the four groups (ϰ2-value=69.50, p=0.0001). **Table 2** reveals gender-wise distribution of patients showing that 64 % of the patients in group A, 96 % in group B and 84 % in group C and D each were males while 36 % of the patients in group A, 4 % in group B and 16 % in group C and D each were females. By using chi-square analysis, statistically significant difference was found, again, as far as gender of the patients was concerned in all four groups (ϰ2-value=8.94, p=0.030). **Table 3** reveals habit-wise distribution of patients showing that 16 % of the patients in group A, 68% in group B, 96 % in group C and 36 % in group D had a history of gutka chewing while 4 % of the patients in group A and C each and 8 % in group D had history of betel nut chewing. Furthermore, 4 % of the patients in group A and 32 % of the patients each in group B and group D had history of smoking and 24 % of the patients in group D had history of alcohol consumption. Also, 76 % of the patients in group A had no habit. Analyzing the findings using chi-square test revealed statistically significant difference in relation to the habit-wise distribution of the patients in all four groups (ϰ2-value=107.60, p=0.0001). **Table 4** reveals the descriptive statistics comparing mean serum ceruloplasmin levels in the four groups. The mean serum ceruloplasmin level in the patients of group A was found to be 43.19 ± 1.90 while in group B, it was 47.68 ± 1.51, in group C, it was 47.74 ± 1.45 and in group D, it was found to be 47.73 ± 0.74. By using one way ANOVA, statistically significant variations were found in the values of mean serum ceruloplasmin levels in the patients of the four groups (F-value=59.58, p=0.0001). On comparing the mean serum ceruloplasmin levels in the patients of the four groups, statistically significant difference was found in all the four groups ($p < 0.05$) except for in group B versus group C, group B versus group D and group C versus group D wherein the variations in the values obtained were not found to be statistically significant ($p>0.05$).

Discussion

Neoplasms are usually preceded with a disordered cellular behavior at the early enough stages and are characterized by alteration of serum glycoproteins and numerous cell surface glycosylation changes at the cellular levels before being detectable clinically and histo-pathologically. An early detection and treatment of PMELs and oral cancers not only reduces the mortality but, also, renders quality life to the survivors. A majority of the neoplasms are preventable as well as curable if they are detected in the early enough stages, especially, PMELs like oral leukoplakia and OSMF, which usually precede frank oral cancers and are amenable to treatment if detected early [15]. Tumor markers, released either by the tumor or, the host cells into the serum of the patient while combating the tumor provide a non-invasive means of such early diagnosis which facilitates an early detection of PMELs or, malignant conditions in their initial stages

Table 1 Age-wise distribution of patients.

Age Group	Group A	Group B	Group C	Group D	ϰ2-value
20-29 yrs	16 (64%)	2 (8%)	9 (36%)	0 (0%)	
30-39 yrs	8 (32%)	6 (24%)	5 (20%)	1 (4%)	
40-49 yrs	1 (4%)	13 (52%)	7 (28%)	6 (24%)	
50-59 yrs	0 (0%)	4 (16%)	4 (16%)	16 (64%)	
≥60 yrs	0 (0%)	0 (0%)	0 (0%)	2 (8%)	69.5
Total	25 (100%)	25 (100%)	25 (100%)	25 (100%)	p-value=0.0001
Mean	28.36	41.32	36.52	53	
SD	5.79	8.02	10.27	5.46	
Range	20-40	28-55	22-55	39-60	

Table 2 Gender-wise distribution of patients.

Gender	Group A	Group B	Group C	Group D	ϰ2-value
Male	16 (64%)	24 (96%)	21 (84%)	21 (84%)	8.94
Female	9 (36%)	1 (4%)	4 (16%)	4 (16%)	p-value=0.030
Total	25 (100%)	25 (100%)	25 (100%)	25 (100%)	

Table 3 Habit-wise distribution of patients.

Habit	Group A	Group B	Group C	Group D	ϰ2-value
Gutka	4 (16%)	17 (68%)	24 (96%)	9 (36%)	
Betel nut	1 (4%)	0 (0%)	1 (4%)	2 (8%)	
Smoking	1 (4%)	8 (32%)	0 (0%)	8 (32%)	107.60
Alcohol	0 (0%)	0 (0%)	0 (0%)	6 (24%)	p-value=0.0001
No Habit	19 (76%)	0 (0%)	0 (0%)	0 (0%)	
Total	25 (100%)	25 (100%)	25 (100%)	25 (100%)	

Table 4 Comparison of Serum Ceruloplasmin levels in the groups: Descriptive Statistics.

Group	N	Mean	Standard Deviation	Standard Error	Minimum	Maximum
Group A	25	43.19	1.9	0.38	40	45.9
Group B	25	47.68	1.51	0.3	42	49.5
Group C	25	47.74	1.45	0.29	44.7	49.7
Group D	25	47.73	0.74	0.14	46.1	48.9
One-way ANOVA						
Source of variation	Sum of Squares	df	Mean Square	F-value	p-value	
Between Groups	383.58	3	127.86	59.58	0.0001	
Within Groups	206.006	96	2.14			
Total	589.58	99				
Multiple Comparisons: Tukey's Test						
Group		Mean Difference (I-J)	Standard Error	p-value	95% Confidence Interval	
					Lower Bound	Upper Bound
Group A	Group B	4.48	0.41	0.0001	3.4	5.56
	Group C	4.54	0.41	0.0001	3.46	5.63
	Group D	4.53	0.41	0.0001	3.45	5.61
Group B	Group C	0.06	0.41	0.999	1.01	1.14
	Group D	0.05	0.41	0.999	1.03	1.13
Group C	Group D	0.01	0.41	1	-1.09	1.07

ensuring an early enough intervention that may eventually affect the prognosis of these lesions.

Cell membrane mainly consists of glycoproteins and glycolipids. Glycoproteins are protein-carbohydrate complexes in which oligosaccharides and or, polysaccharides are joined to specific amino acids of proteins by covalent linkages [15]. There have been a plethora of studies that have developed and successfully demonstrated the use of such changes as potential markers in the diagnosis and management of oral cancers. The present study evaluated the diagnostic role of serum ceruloplasmin levels as a potential biomarker in the early detection of oral potentially malignant epithelial lesions (PMELs) including oral leukoplakia and OSMF and OSCC patients.

In the present study, the age of the patients in the control group (A), patients with oral leukoplakia (B), OSMF (C) and OSCC (D) ranged from 20-40 years, 28-55 years, 22-55 years and 39-60 years respectively, thus, showcasing a broad range of probability of the occurrence of PMELs and OSCC in the affected individuals. The mean age for OSCC (D) was found to be 53 years indicating the peak incidence of occurrence for OSCC in the 5th decade of life, thus, highlighting the significance of and the role ageing plays in the causation of OSCCs with decreasing immuno-surveilance which was in accordance with the findings of the studies conducted by Pradeep MR et al [16] and Shetty RK et al [17] in which the mean age for the occurrence of OSCC was found to be 51 years and 55.92 ± 10.17 years respectively.

Furthermore, there are varying reports on sex ratio for the occurrence of PMELs and OSCC in the different studies published. In the present study, out of the 25 oral leukoplakia patients, 24 (96 %), 25 OSMF patients, 21 (84 %) and 25 OSCC patients, 21 (84 %) of the patients were males while 1 (4 %), 4 (16 %) and 4 (16 %) of the patients were females respectively indicating a male predominance in all the four said groups. Again, the male-female ratio in the OSCC group in the present study was found to be 5.25:1 which was similar to the findings of the study conducted by Shetty RK et al [17] who found a higher male prevalence reported for the said lesions with the male-female ratio being 5:1 in their study. Likewise, in the similar other studies conducted by Elango JK et al [18] and Mehrotra R et al [19], the male-female ratio was found to be 4:1 while the mean age of the patients was seen to be 55.92 ± 10.17 years. The occurrence of PMELs and oral cancers is seen to be higher in the males and this might be due to the much prevalent habit of chewing gutkha, betel nut, smoking and drinking alcohol etc. in males as compared to the females.

Literature is abuzz with correlation of oral malignancies and habits such as smoking and tobacco chewing. Gutkha chewing (36 %) and smoking (32 %) were the most common habits found in the present study followed by alcohol (24 %) and betel nut chewing (8 %). Thus, guthka chewing, smoking and alcohol were found to be the major risk factors for the causation of oral PMELs and OSCCs in the present study. Similar risk factors in head and neck malignancies have been reported in the various other studies including the ones conducted by Shashikant MC and Rao BB [20] and Day GL and Blot WJ [21].

Oxidative stress is an imbalance between the free radical damage and the antioxidant protection in the body. A balance between oxidant carcinogens and endogenous antioxidant defenses is of particular relevance to the process leading to the evolution of carcinogenesis. Copper and ceruloplasmin have been observed to be significantly increased in numerous cancers [22]. Cupric ions are reported to inhibit the production of singlet oxygen and this is of particular significance because of the latter's ability to cross the cell membrane and its high reactivity towards the various biomolecules [23] while ceruloplasmin is a glycoprotein encoded by CP gene on chromosome no.3q24 involved in the transport of copper ions in the body and also, in iron metabolism by virtue of its ferroxidase activity. It is synthesized primarily in the liver

and contains 6 to 7 copper ions and is a well-known acute phase reactant and a transport protein [24].

In the present study, sera levels of ceruloplasmin levels showed significantly higher levels in all the three groups including oral leukoplakia (B), OSMF (C) and OSCC (D) with the corresponding values being 47.68 ± 1.51 mg/dl, 47.74 ± 1.45 mg/dl and 47.73 ± 0.74 mg/dl respectively than as compared to the controls (A) being 43.19 ± 1.90 mg/dl. The results of the present study revealed that as compared to the controls (A), ceruloplasmin levels remained significantly ($p < 0.05$) high in all the three study groups, though, were found to be nearly constant in the said groups including oral leukoplakia (B), OSMF (C) and OSCC (D) in accordance with the findings of the studies conducted by [25,26] which observed a better correlation between elevated serum copper levels and some malignancies. This was probably the first evidence which created ceruloplasmin as a potential candidate to be used as a biomarker for oral potentially malignant epithelial lesions (PMELs) including oral leukoplakia and OSMF and OSCC patients. Similar correlation was found between increased sera copper levels and areca nut chewing habit in yet another study conducted by Arakeri G et al [27].

Also, the mean sera ceruloplasmin levels of 43.19 ± 1.90 mg/dl in the controls (A) as found in the present study were slightly lower when compared to an earlier study conducted by Singh M et al [28] who found the mean sera ceruloplasmin levels in the controls to be 49.06 ± 6.83 mg/dl in their study. Likewise, numerous other studies conducted by Varela AS et al [29] and Abbas AW et al [30] reported significantly reduced mean sera ceruloplasmin levels of 32.4 mg/dl and 31.1 mg/dl in the controls in their studies respectively. In the present study, the mean sera ceruloplasmin levels were significantly ($p < 0.05$) higher in the OSCC (D) group as compared to the controls, the level of change, though, with respect to the other PMELs, oral leukoplakia (B) and OSMF (C) and OSCC (D), did not represent any significant variation.

Jayadeep A et al [31], also, reported similar findings in relation to the mean sera ceruloplasmin levels in their study on oral pre-cancerous lesions and frank oral cancers highlighting the diagnostic significance serum ceruloplasmin levels can have not only in the early detection of oral potentially malignant epithelial lesions (PMELs) and frank oral cancers but the prognostic significance it can have in marking the disease progression. The findings of the present study were, also, in close accordance with the findings of the yet another study conducted by Singh M et al [28] which, also, reported significant rise in the mean serum ceruloplasmin levels in the OSMF, oral leukoplakia and OSCC patients with the corresponding values being 102.16 ± 16.33 U/L, 93.04 ± 16.56 U/L and 107.08 ± 16.76 U/L respectively. The mean serum ceruloplasmin level in the controls, though, was recorded to be on the higher side in their study being 49.06 ± 6.83 U/L as compared to the present study wherein it was found to be 43.19 ± 1.90 U/L. The increased serum ceruloplasmin levels, though, can be attributed to the elevated antioxidant activity of the serum in such cases [32].

Contrary to the findings of the present study, though, Anuradha CD and Devi CS [33], observed a significant decrease in the mean serum ceruloplasmin and copper levels ($p < 0.02$ and $p < 0.001$ respectively) in their study in all the patients in various stages of OSMF in contrast to the present study wherein the mean serum ceruloplasmin levels showed a significant increase in the patients of OSMF (C) as compared to the controls (A). The decreased levels of copper in the patients of OSMF, though, can be attributed to the consumption of copper ions for the activation of enzyme lysyl oxidase, an enzyme that plays a significant role in the causation of OSMF by affecting collagen metabolism leading to an increased fibrosis of the oral submucosal tissues. The decrease in copper levels obviously bring about a decrease in the mean serum ceruloplasmin levels, too, influencing iron absorption and mobilization of iron from the liver and other tissue stores, further, leading to iron deficiency anemias, an important finding in the patients of OSMF [34].

In accordance with the findings of the present study, Akinmoladun VI et al [35], though, also, found increased mean serum ceruloplasmin levels in patients with oral PMELs and frank oral cancers which were recorded to be statistically significant ($p < 0.01$) compared to the controls in their study. Increased serum ceruloplasmin levels have, also, been previously reported in various malignancies with the reason behind the same being explained on similar oxidant-antioxidant cellular disturbances in the host [30,36,37].

Even though not being sensitive for phase base reactions, usefulness of serum ceruloplasmin levels never becomes less as could be seen from the findings of the present study where its sensitivity and specificity had been benchmarked in the various oral PMELs and frank OSCCs. Also, it is noteworthy that serum ceruloplasmin is expressed by the tumoral cells just as the other established biomarkers like carcino-embryogenic antigen (CEA) and hence, makes a featured marker of future use in such situations when investigated in detail. Similar conclusions were drawn in the studies conducted by Kunapuli SP [24] and Abd-El-Fattah M et al [38] who highlighted its significance to be used as a potential biomarker in the early detection of oral potentially malignant epithelial lesions (PMELs) and frank oral cancers. Likewise, Andrzejewska H et al [39], also, reported the said correlation between serum ceruloplasmin levels and the various clinical stages of the cancer of larynx while Krecicki T and Leluk MV [40] concluded from their study that determination of serum ceruloplasmin levels could be of use in the monitoring of cancer patients as well as in assessing the prognosis in such patients.

Limitations and Future Perspectives

There are few possible limitations that need to be addressed as far as the present study is concerned. First, the serum ceruloplasmin levels were not correlated with the histopathological grading and staging of the included oral PMELs and OSCC. Second, serum ceruloplasmin levels, though, found to be significantly higher in the oral PMELs and OSCC as compared to the controls, inter-group variations were reported to be nearly constant in all the three study groups indicating no significant difference in the transition of oral PMELs into frank malignant lesions. The major reasons behind the said variations to be insignificant

might be the smaller sample size as well as the specificity and sensitivity of the kit used in the present study. Third, a plethora of reasons have been cited for an increase or, decrease in the mean serum ceruloplasmin levels including the various acute and chronic inflammatory processes, active hepatitis, liver and biliary cirrhosis, copper toxicity, zinc deficiency and various malignant tumors including the leukemias and Hodgkin's lymphomas. It becomes imperative, therefore, to detect the correct cause for the increase in serum ceruloplasmin levels and thus, renders a need to pre-evaluate the hepatic and renal diseases in future research projects. Also, post-treatment serum ceruloplasmin levels were not evaluated in the present study. Therefore, their impact on the prognosis of the lesions, post-treatment, cannot be commented-upon. The researches focusing on serum ceruloplasmin, though, being in the very primitive stages, mandate the need for further researches to be conducted in this regard to justify its contribution as a specific biomarker for oral PMELs and frank oral cancers.

Conclusion

Serum ceruloplasmin levels were found to be raised in all the three groups including oral leukoplakia, OSMF and OSCC as compared to controls. The inter-group variations reported, though, were found to be nearly constant indicating no significant difference in the transition of oral PMELs to frank malignant lesions. Likewise, when the patient's age and sex were considered, a broad range of probability of occurrence was evidenced with the highest incidence recorded for OSCC in the 5thdecade of life and a specific male predilection for all oral PMELs and oral cancers. Also, a definitive association was found between the harmful habits and oral PMELs and oral cancers in decreasing order for gutkha chewing, smoking, alcohol consumption and betel nut. Thus, it could be concluded from the observations of the present study that serum ceruloplasmin, in conjunction with clinical diagnostic procedures, can be used as a potentially reliable serological biomarker in the early detection of oral PMELs and frank oral cancers

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