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ESTIMATION AND CORRELATION OF α-AMYLASE LEVELS AS SALIVARY DIAGNOSTIC BIOMARKERS IN SMOKELESS TOBACCO USERS WITH PERIODONTITIS – A BIOCHEMICAL ANALYSIS.

Pujara M[1*], Gupta S[2], Patel V[3], Patel J[4]

[1*]Dr. Meghna Pujara Senior Lecturer, Department of Periodontology, Faculty of Dental Science, Dharmsinh Desai University, Nadiad

[2]Dr. Shalini Gupta, Professor, Department of Periodontology, Faculty of Dental Science, Dharmsinh Desai University, Nadiad

[3]Dr. Vasumati Patel, Professor & Head, Department of Periodontology, Faculty of Dental Science, Dharmsinh Desai University, Nadiad

[4]Dr. Jinal Patel, Senior Lecturer, Department of Periodontology, Faculty of Dental Science, Dharmsinh Desai University, Nadiad

***Corresponding Author:**Dr. Meghna Pujara,

*****Senior Lecturer, Department of Periodontology, Faculty of Dental Science, Dharmsinh Desai University, Nadiad, Address: L-103, AnandiLyf, Nr. Vaishnodevi Underbridge, S.P. ring road, Tragad, Ahmedabad, Gujarat

Abstract

Aims: The purpose of this study was to estimate and correlate the levels of α-amylase as salivary biomarkers in healthy and smokeless tobacco users with periodontitis.

Methods and Material: A total of four hundred and forty four subjects fulfilling the inclusion and exclusion criteria were selected and divided into four different groups in this cross-sectional study and 5 ml of unstimulated saliva was collected by spitting method and sent for biochemical analysis to estimate levels of α-amylase.
Statistical analysis used: Descriptive statistics, One-way ANOVA, Tukey's post-hoc tests and Pearsons correlation.

Results: The data presents the mean value of α-amylase in control group, test group 1, test group 2 and test group 3, as 453.66±162.81, 864.83±324.92, 683.99±309.06, 1078.37±750.56 (IU/L)×10^2) respectively. Multiple intergroup comparisons showed statistically significant result with p value <0.001. The salivary α-amylase levels increased in subjects with periodontitis compared to the control group. The salivary α-amylase levels were higher in smokeless tobacco users with periodontitis. The correlation between the parameters α-amylase (IU/L)×10^2) & Nicotine dependence score showed a poor positive correlation and was significant with a p value of <0.001.

Conclusions: The results of this study related to salivary α- amylase as salivary Biomarker lead us to conclude that estimation of salivary α- amylase could be a prognostic biomarker in identifying the patients with higher risk at a very early stage. The method being noninvasive, inexpensive, and simple can be used as a screening test and can help in educating patients about the detrimental effects of tobacco.

Key-words: Salivary α- amylase, Smokeless tobacco, Periodontitis, Biomarkers, Nicotine

Key Messages: Salivary α- amylase promises to be a potential prognostic biomarker in early detection of periodontal disease as well as can contribute significantly in assessing the potential for malignant transformation with reasonable accuracy.

Introduction:

Periodontal disease is a multifactorial disease which is initiated by plaque and influenced by other factors which contribute in the pathogenesis and progression of disease.[1]Although periodontal diseases are caused primarily by dental plaque, risk factors like tobacco consumption can modify the periodontal response to microbial aggression.[2] The two means of tobacco usage products namely smoking bidis, cigarettes, etc. and smoke-free tobacco products have been studied and examined throughout the world and the practice of smoke-free/smokeless tobacco is common. However, smokeless tobacco use has been found to have detrimental effects on periodontal health.

Smokeless tobacco (SLT) is defined as a product that contains tobacco, is not smoked or burned at the time of use, and commonly consumed orally or nasally. The tremendous and widespread use of SLT dates back to the early 16th century due to its recognized properties of increasing salivation, reducing thirst and appetite, serving therapeutic purposes and even reducing dependence on smoked tobacco.[3]

Tobacco chewers have an incidence of gingivitis and gingival bleeding that is similar to the incidence among non-users. SLT is commercially available as gutka, containing areca nut, slaked lime and spices and more nicotine content than cigarette.[4] India sums up for 70% of the global burden of smokeless tobacco.[5]Pindborg (1947) was one of the torch bearers to examine the connection between tobacco use and periodontal disease.[6]

Periodontitis is the most prevalent chronic inflammatory conditions affecting the adult population. The activation of patient's host response liberates a myriad of metabolic byproducts at the interface between the tooth and the periodontal pocket which results into liberation of destructive cellular enzymes, cytokines, chemokines, and other mediators of tissue destruction.[7] There are several markers in saliva which have been proposed and used as diagnostic tests for periodontal disease but the diagnostic tests should demonstrate sensitivity and specificity.

Periodontal disease has been primarily diagnosed by clinical and radiographic findings. The saliva contains a wide and unique variety of proteins and enzymes with important oral biological functions. Emerging evidence suggests that the estimation and correlation of salivary biomarkers can serve as valuable indicators of periodontal disease severity and progression. Saliva, as a diagnostic fluid, contains various biomarkers that reflect the local and systemic changes occurring in response to periodontal disease. Among these biomarkers, alpha amylase (α-amylase) has shown promise in assessing periodontal tissue damage and inflammation.Salivary amylase has been investigated in numerous behavioral studies as a potential non-invasive and sensitive biomarker of acute stress-induced activity of the sympathetic nervous system.[8] α- amylase, is involved in the breakdown of starch and is seen to be increased in saliva during periods of stress and inflammation.

The effect of smokeless tobacco (SLT) has attained remarkably less attention in the etiology of periodontal disease compared to smoking. There is no study done in the literature to assess and compare periodontal status of smokeless tobacco users and non-users in association with salivary α-amylase levels. Hence this study was done to estimate and compare the salivary α -amylase levels of smokeless tobacco users and non-users with their periodontal status.

The study involved a comprehensive analysis of salivary samples from smokeless tobacco users with periodontitis, comparing their biomarker levels to non-tobacco users with periodontitis as well as healthy individuals without any periodontal disease. By analyzing salivary biomarkers, we aimed to determine if there was a significant difference in α- amylase levels among the different groups, consequently emphasising the potential role of smokeless tobacco in exacerbating periodontal inflammation and tissue destruction

Subjects and Methods:

This cross-sectional study was carried out in the Department of Periodontology, Faculty of Dental Science, Dharmsinh Desai University, Nadiad, after attaining approval from the institutional ethical committee. A total of 444 subjects were included in the study. The subjects excluded from the study were, those who had no history of systemic disease, smoking, usage of any antibiotic/anti-inflammatory medication and corticosteroid therapy for 3 months prior to study, regular usage of mouthwash, alcohol consumption and periodontal therapy 6 months prior to study. Subjects with dry mouth conditions, those undergoing orthodontic treatment, pregnant, menopause or lactating women, and who had used chewing gum before the saliva collection were also excluded from the study.

Sample size:

Total 444 subjects were included in the study having age in the range of 18-55 years. They were divided into following groups with required sample in each group were 111 subjects based on the power analysis having 1% alpha error, 95% power of the study and a clinically significant difference of 50 units.

Group division:

Control Group (n=111): Periodontally healthy subjects with sulcus depth ≤3mm with no attachment loss and Bleeding on Probing (BOP) (as per gingival index by Loe and Sillness 1963).
Test Group 1 (n=111): Periodontally healthy subjects with sulcus depth ≤3mm with no attachment loss and Bleeding on Probing (BOP) (as per gingival index by Loe and Sillness 1963) with smokeless tobacco using habit.
Test Group 2 (n=111): Generalized periodontitis subjects with periodontal pocket depth ≥5mm as well as clinical attachment loss involving minimum 30% sites with BOP without smokeless tobacco consuming habit.
Test Group 3 (n=111): Generalized periodontitis subjects with periodontal pocket depth ≥5mm as well as clinical attachment loss involving minimum 30% sites with BOP having smokeless tobacco consuming habit.

Determination of nicotine dependence:

Nicotine dependence was determined on the basis of Fagerstrom Nicotine Dependence Scale – Smokeless Tobacco (FTND-ST).[9] A score of 5 or more indicated a significant dependence, while a score of 4 or less showed a low to moderate dependence.

Collection of saliva sample:

Subjects who agreed were included in the study and written consent was obtained from the patients for the study. After a detailed dental examination the subjects were alloted either in Control group, Test group 1, Test group 2 or Test group 3. The subjects were rescheduled next day in morning from 9:00 am to 12 noon, atleast one hour

after the last meal to standardize the collection according to the circadian rhythm. The subjects were asked to rinse thoroughly with distilled water before the collection of salivary sample. Five minutes after the oral rinse the subjects were asked to swallow any residual saliva that might be in their mouth and were refrained from talking and were asked to drop down the head and not to cough up mucus as saliva was collected. Then the subjects were asked to let the unstimulated saliva pool in their floor of the mouth to their maximum extent and then expectorate into the container every 60 seconds for 10 minutes or when the subject experienced an urge to swallow the fluid accumulated in the floor of the mouth till the desired 5 ml quantity was collected to evaluate the salivary α-amylase. The saliva samples were transported to the laboratory for estimation immediately using standard gel coolant pack in order to maintain the temperature between 2 ºC to 4 ºC. Biochemical assay of saliva samples were carried out using Amylase in vitro diagnostic kit (AGAPPE),in the Department of Biochemistry, Dr. N D Desai Medical College, Dharmsinh Desai University, Nadiad, Gujarat, India.

Biochemical analysis of salivary α-amylase:

1:100 diluted salivary sample was prepared by adding 990 µl distilled water along with 10 µl of collected salivary sample using calibrated micropipette (Figure 1). 1000 µl of reagent was taken in test tube 3 with the help of calibrated micropipette, to which 25 µl of prepared diluted salivary sample was mixed (Figure 2) and then incubated at normal room temperature for 1 minute. Prepared solution was analyzed in semi automatic biochemical analyzer machine (Erba Mannheim Chem 5x) from which digital readings were obtained and recorded (Figure 3).

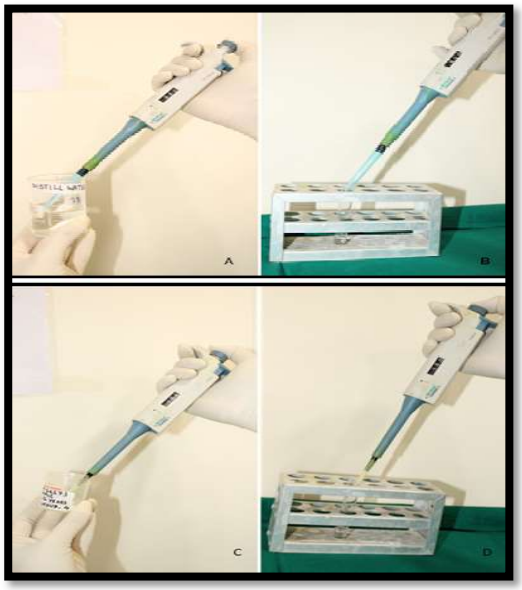


Figure 1: Preparation of 1:100 diluted salivary sample

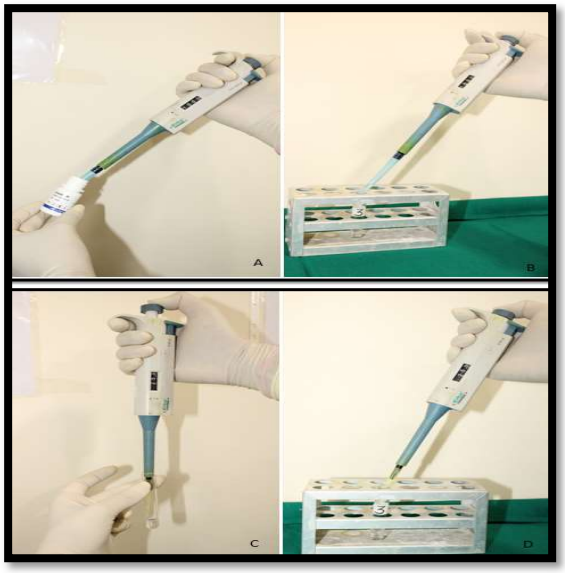


Figure 2: Prepared diluted salivary sample added to the reagent

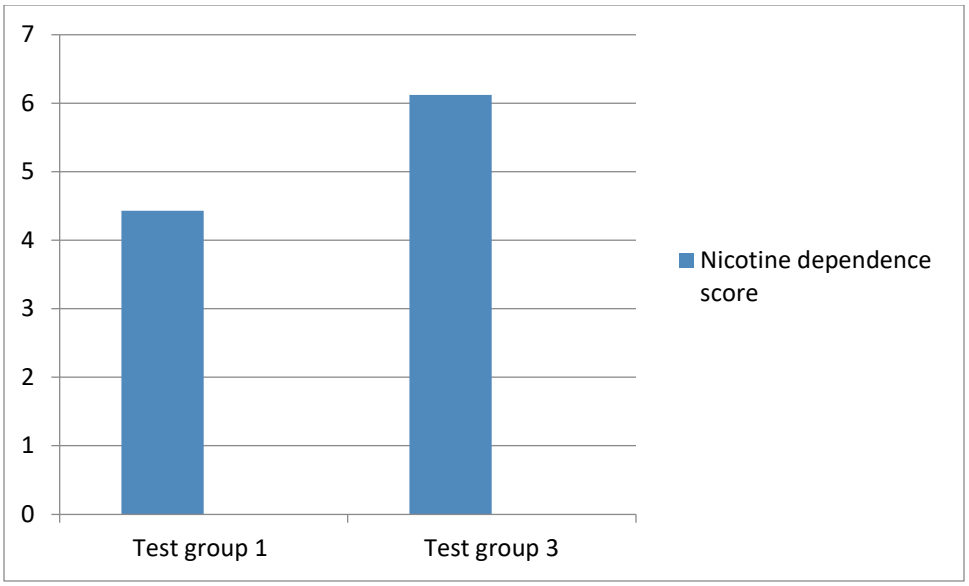
Figure 3: Prepared solution analyzed in semi automatic biochemical analyzer machine (Erba Mannheim Chem 5x)

Statistical Tests:

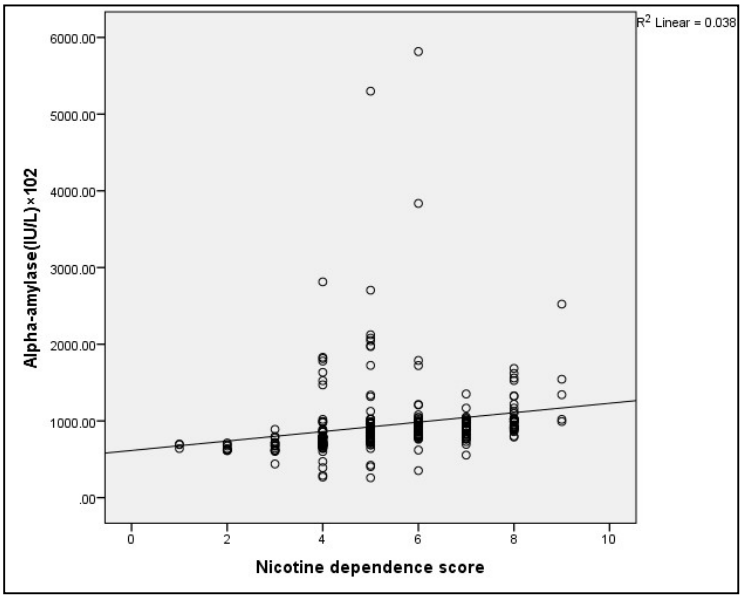
Apart from Descriptive Statistics, one way ANOVA with Tukey Post Hoc was used to assess the difference between the levels of salivary α-amylase with respect to different groups. Pearsons correlation was done to correlate the nicotine dependence score and the other variables in total. The statistical analysis was undertaken at 95% confidence level with statistical significance at p value less than 0.05.

Results:

Mean values of Nicotine dependence score distribution amongst periodontally healthy subjects (Test group 1), and subjects with periodontitis (Test group 3) with smokeless tobacco using habit are 4.43±1.5 and 6.12±1.26 respectively as shown in Graph 1.



Graph 1: Mean values of Nicotine dependence score



Graph 2: Pearsons correlation to correlate the Nicotine Dependence score and Alpha Amylase levels

The mean value of α-amylase in control group, test group 1, test group 2 and test group 3 in this study was found to be 453.66±162.81, 864.83±324.92, 683.99±309.06, 1078.37±750.56 (IU/L)×10^2) respectively as shown in Table 1. Multiple intergroup comparisons showed statistically significant result with p value <0.001 as shown in Table 2. The salivary α-amylase levels increased in subjects with periodontitis compared to the control group. The salivary α-amylase levels were higher in smokeless tobacco users with periodontal disease. The correlation between the parameters α-amylase (IU/L)×10^2) & Nicotine dependence score showed a poor positive correlation (Graph 2), and was significant with a p value of <0.001 as shown in Table 3.

	Age (years)	Alpha-amylase(IU/L)×10^2
Control(111)	25.91±4.95	453.66±162.81
Test Group 1(111)	24.94±5.15	864.83±324.92
Test Group 2(111)	41.91±7.65	683.99±309.06
Test Group 3(111)	45.32±5.28	1078.37±750.56
F	271.578	71.187
P value	<0.001	<0.001

Table 1: One way ANOVA shows P value <0.001

Parameter compared using Tukey HSD	**Comparison of**	**Comparison with**	**mean difference**	**standard error**	**P value**
Alpha-amylase(IU/L)×10^2	Control	Test Group 1	-411.16329*	52.86234	<0.001
		Test Group 2	-230.32643*	52.39447	<0.001
		Test Group 3	-624.71081*	52.74261	<0.001
	Test Group 1	Test Group 2	180.83686*	52.515	**0.008**
		Test Group 3	-213.54752*	52.86234	**0.001**
	Test Group 2	Test Group 3	-394.38438*	52.39447	<0.001

Table 2: Posthoc analysis

SNO	PARAMETERS BEING CORRELATED	N	Correlation(r)	P VALUE
1	Alpha-amylase(IU/L)×10^2& Nicotine dependence score	333	0.196	**<0.001**
2	Nicotine dependence score & Probing depth (test group 2)	111	-0.034	0.72
3	Nicotine dependence score & Clinical Attachment Loss (test group 3)	111	0.358	**<0.001**

Table 3: Pearsons correlation to correlate the Dependence score and the other variables in total

Discussion:

Tobacco is a foreign substance,[10] shown to stimulate[11] the body to produce more free radicals that are endogenously produced in various cellular metabolic activities and which play a role in preventing microbial pathogen invasion at low concentrations. However, as their concentration rises, they may damage cellular components, eventually causing denaturation or mutation, which can be seen in parasitic infections, inflammatory diseases and cancer.[12]Smokeless tobacco products are highly addictive due to their high nicotine content. Subjects with pre-malignant disease were excluded in our study to evaluate any biochemical changes that can be identified in an early stage and also to identify the patients at increased risk in which preventive measures can be instituted. Periodontal disease is a chronic bacterial infection characterized by lasting inflammation, connective tissue

breakdown, and alveolar bone destruction.[13]They are multifactorial conditions that are affected by both genetic and environmental factors.

With the emerging need for the non-invasive diagnostic approaches, biochemical tests are introduced and are highly preferred tool for diagnosis and treatment planning in relation to diseases that have an obvious metabolic basis and those in which biochemical changes are a consequence of the disease. Advancements in oral and periodontal disease diagnostic research are, therefore, proceeding toward methods whereby periodontal risk can be identified and quantified by objective measures such as biomarkers. Human saliva is similar to blood in various biological aspects. Also, it possesses a simple and non-invasive collection method with low-cost and easy storage nature when compared with gingival crevicular fluid (GCF).

Periodontal destruction has been seen more in tobacco chewers when compared to smokers and non-tobacco users. This may be due to the cumulative effect of placement of tobacco for longer duration in the mouth and also more irritants seen in smokeless tobacco products.[14]

Salivary α-amylase has been used as a biomarker for stress that does not require drawing of blood.[15] Certain studies argue that α-amylase as a biomarker is also associated with periodontal inflammatory disease.[16,17]Tobacco consumption modifies several biological parameters, including α-amylase.[18] Hence, the present study was planned to estimate and compare the level of salivary alpha-amylase in periodontally healthy subjects and patients with periodontitis with and without smokeless tobacco chewing habit.

Previous studies carried on tobacco dependence's impact on the activity of serum and salivary alpha-amylase resulted in divergent viewpoints. For Nater et al.,[19] smoking tobacco elevates the value of alpha-amylase activity in serum and saliva. On the contrary, for other authors, smoking tobacco does not affect the value of serum and salivary alpha-amylase activity.[20-22] Nevertheless, investigations on the influence of smokeless tobacco consumption on alpha-amylase activity are very few. The research work done by Reddy et al.[23] reported increased levels of salivary alpha-amylase activity in tobacco chewers.

The present study is in agreement with the study conducted by Pooja Bharadwaj et al.,(2020)[24] who evaluated and compared the levels of α-amylase in serum and saliva between chronic periodontitis and healthy patients. Mean salivary α-amylase level ($p < 0.001$) was highly significant in chronic periodontitis group as compared to healthy control group while there was no significant difference in serum α-amylase level ($P > 0.05$) between chronic periodontitis group and healthy control group. G A Sánchez et al., (2011)[25] reported salivary α-amylase level in health as 89.63±11.0 U/ml, moderate periodontitis as 122.52±6.8 U/ml, and severe periodontitis as 136.94±11.2U/ ml. Henskens YMC (1996)[26] compared salivary α-amylase level in 25 healthy and 25 chronic periodontitis subjects by enzyme-linked immunoabsorbent assay technique and found levels of salivary α-amylase to be 76±43 U/ml and 155±103 U/ml respectively. The present study is also in agreement with the studies carried out by Kejriwal S (2014),[27] Anuradha Kanhere et al.,(2017).[28] Maier et al. (1991)[29] found increased salivary α-amylase activity after intravenous nicotine infusion (20 μg/kg). Jauniaux et al. (1999)[30] observed higher α-amylase activity in fetal plasma of smoking mothers.

The increase in salivary α-amylase may be due to the response of salivary glands to inflammatory diseases like periodontitis resulting in increased synthesis and secretion of α-amylase so as to enhance the oral defense mechanism.[31]Studies showed that α-amylase is a major lipopolysaccharide binding protein of Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis and interferes with bacterial adherence and biofilm formation.[32]Thus, the high concentration of salivary α-amylase seen in present study suggests it to be a significant

defense molecule essential for the innate immunity in the oral cavity. Increased leakage of plasma proteins into saliva due to inflammation could also be responsible for elevated salivary α-amylase levels.[33]

Contrary to this study, Debasis Biswas et al., (2023)[34]found the baseline amylase activity of normal subjects varying between 126 and 160 U/ml whereas the activity in the tobacco-users ranged from 99 to 126 U/ml. When the mean baseline activity was compared, a significant decrease in amylase activity ($P<0.0001$) was observed in tobacco-user subjects. Masood H Khan et al., (2018)[35]conducted a study and calculated salivary amylase levels in smokers, chewers and controls where, amylase level was significantly reduced in both tobacco chewers and smokers. Moutawakilou Gomina et al., (2013)[36] concluded that the mean activity of serum and salivary α-amylase was substantially higher in tobacco non-consumers than in chewers ($p=0.01$ and 0.02, respectively). The decreased levels may owe to the injury to ductal secretory unit caused by tobacco related toxic products or the effect of increase salivary flow with dilutional effect in tobacco users. There is excess secretion of saliva in tobacco chewers, as chewing may lead to hypertrophy of the masticatory muscles which may express greater salivary flow from the glands.

In the present study we found increase in the mean value of Nicotine dependence score from periodontally healthy to periodontitis subjects, also there was a decrease in probeable probing depth and increase in clinical attachment loss in periodontitis subjects with smokeless tobacco using habit compared to subjects with periodontitis without smokeless tobacco using habit . This finding was in agreement with the study by, Najith Amarasena et al.,[37]Jyoti Goyal et al.,[38]Dr. Sirjana Dahal et al.,[39]where nicotine dependence was found to be significantly correlated with periodontal status and loss of attachment among nicotine dependent individuals attending community dental camps. Exposure to high nicotine concentration causes alteration in gingival blood flow, gingival crevicular fluid, connective tissue turnover, and cell function of periodontium leading to increased pocket depth, loss of periodontal attachment, alveolar bone height, and higher rate of tooth loss.

Limitations:

Despite the cost and noninvasive benefits of salivary biomarkers analysis, there has been an unfortunate incidence of interferences that are associated with insufficient volume of the sample of saliva collected, reduced sensitivity of salivary concentration of biomarkers, and early expiration of reagents used in the salivary biomarkers analysis that affect the reaction and lead to a false-positive or false-negative results.

Conclusion:

The results of this study along with the observations of the studies performed in the past related to salivary α-amylase as salivary biomarker lead us to conclude that estimation of salivary α- amylase could be a prognostic biomarker in identifying the patients with higher risk at a initial stage. The knowledge about the potential role of salivary biomarkers could contribute to the development of targeted interventions and treatments for individuals affected by smokeless tobacco use. Further studies arerequired to explore the full potential of salivary biomarkers in contemporary diagnosis at a larger scale which is a groundbreaking field having diagnostic and therapeutic potentials.

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