

## Correlation of Salivary Cotinine Levels with Severity of Periodontitis

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**Abstract:** Periodontitis is a multifactorial disease. There are different risk factors for periodontal disease. Amongst them tobacco smoking is a well established risk factor. Chronic exposure to tobacco and its by products significantly affects the prevalence and progression of periodontal diseases. Smoking is an established and modifiable risk factor for periodontitis. Cotinine, the major proximate metabolite of nicotine, widely used as a biomarker of tobacco exposure. The purpose of this study was to assess salivary cotinine levels in smokers and non-smokers with chronic periodontitis and also to assess the level of nicotine dependence in smokers. In this cross sectional study a total of 90 male patients with a age group of 30-60 years with chronic periodontitis were selected for the study. The patients were divided into two groups. Group A consisted of 45 patients (non-smokers) and Group B consisted of 45 patients (smokers). The salivary cotinine levels were assessed by collecting salivary samples of all the patients using ELISA. There was a statistically significant difference in PPD (5.6, 4.3mm), CAL (5.3, 4.2mm), GI (0.86, 1.4) and GBI (8%, 43%) values between smokers and non smoker groups respectively. But the plaque index showed no statistically significant difference between smokers and non-smokers (1.4, 1.4). Salivary cotinine levels were significantly higher in smokers group than in non-smokers (42.31, 4.65pg/ml). The overall PPD, CAL was positively correlated with the overall cotinine levels but overall GI, GBI values were negatively correlated. The plaque index values were not significantly correlated. Fagerstrom index for nicotine dependence has showed a positive correlation with the salivary cotinine values in smokers. The increased levels of salivary cotinine have a strong association for increased severity for chronic periodontitis in smokers compared to non-smokers.

**Keywords:** Periodontitis, multifactorial disease, tobacco, smoking

### INTRODUCTION

Periodontitis is a multifactorial disease. There are different risk factors for periodontal disease. Amongst them tobacco smoking is a well established risk factor [1]. Chronic exposure to tobacco and its by products significantly affects the prevalence and progression of periodontal diseases [2,3]. In addition, tobacco use complicates periodontal therapy and substantially reduces the possibility of favourable treatment outcomes. The association is independent of other factors such as oral hygiene and age [4]. Evidence supports that periodontitis is more prevalent in smokers than in non-smokers. Tobacco metabolites suppress neutrophils function, influencing host defence mechanism and inhibit immune responses [5].

Biochemical measure of tobacco use is desirable for determining disease activity and treatment outcome [6]. The exposure of tobacco smoke can be assessed by the biochemical measure of the levels of cotinine which is a proximate metabolite of nicotine. On an average 70-80% of nicotine is converted to cotinine. It can be measured in blood, saliva, urine. Cotinine is formed by cytochrome P450 mediated

oxidation of nicotine. As it is more stable and has a longer half life of average 17hours (Range: 10-30hours) than that of nicotine (2-3hours) and remains relatively constant, so it is considered as an accurate measure of smoking [7].

Cotinine can be measured in a variety of body fluids, including blood, saliva, urine, breast milk, and crevicular fluid. A report by Sepkovic *et al.* [8] in 1986 suggested that elimination of cotinine from saliva is slower than from blood or urine. The study indicated that after cessation of smoking, blood and urine concentrations of cotinine declined to non-smoking levels within three to four days. Salivary concentrations of cotinine decreased from 600 ng/ml to 300 ng/ml in first three days and then showed no further decline for up to one week.

Saliva is preferred source to assess the levels of cotinine as it correlates well with that of serum and also the samples are easy to obtain and more compliant for the patient [9].

ELISA is sensitive but not specific, that means it detects the chemicals that are structurally similar to cotinine but it can be used as a more economical and easier way of estimating the cotinine levels [7].

Gonzalez *et al.* [10] in 1996 measured serum cotinine using ELISA and correlated its level with severity of periodontitis and concluded that higher concentrations of cotinine levels are associated with severity of periodontitis. A 10 year longitudinal study [11] was done to determine the association of salivary and gingival crevicular fluid cotinine levels with periodontal disease status in smokers and nonsmokers by using ELISA and concluded that cotinine levels are stable over time and that levels relate positively and significantly to periodontal disease in smokers with periodontitis. Another study [12] by using ELISA determined the relationship between passive and active smoking on the basis of salivary cotinine and severity of periodontitis and finally concluded that passive smoking classified in terms of salivary cotinine level may be an independent risk indicator.

As saliva collection is non-invasive and levels of cotinine in saliva are similar to those in serum, this study was conducted to correlate the salivary cotinine levels with severity of periodontitis by using ELISA.

The aim of the present study was to correlate the levels of salivary cotinine in smokers and non-smokers with periodontitis and to evaluate the nicotine dependence in smokers.

## METHOD AND MATERIALS

A total of 90 male patients within the age group of 30-60 years with chronic periodontitis attending the department of periodontics, Meghna Institute of Dental Sciences, Nizamabad were selected for the study. 45 patients with no history of smoking in their lifetime were included in Group A (non-smokers) and 45 patients with history of cigarette smoking only were included in Group B (smokers). Criteria for chronic periodontitis was presence of at least two or more teeth with Probing Pocket depth (PPD)  $\geq 5$ mm and Clinical Attachment Loss (CAL)  $\geq 3$ mm [12]. The purpose and procedure of the study was explained to the subjects and an informed consent was obtained. All the subjects enrolled in the study fulfilled the inclusion and exclusion criteria. Permission from the ethical committee was obtained prior to the study. Salivary samples of all the patients were collected in test tubes using funnel. The clinical parameters included were Plaque index [13], Gingival index [14], Gingival bleeding index [15]. Probing Pocket Depth (PPD), Clinical Attachment Loss (CAL) was measured on four surfaces of the tooth.

## Methodology

After selection of subjects, a detailed case history was taken which also included Fagerstrom index for nicotine dependence for smokers group.

## Fagerstrom test for nicotine dependence (FTND) [16]

This is a six item test to diagnose degree of nicotine dependence in smokers.

## Scoring the Fagerstrom Test for Nicotine Dependence (FTND)

In scoring the Fagerstrom Test for Nicotine Dependence, the three yes/no items were scored 0 for no and 1 for yes. The three multiple-choice items were scored from 0 to 3. The items were summed to yield a total score of 0-10.

## Method of collecting saliva (navazesh method) [17] (Figure -1)

Many studies have shown that unstimulated saliva is more preferable than stimulated saliva. The reason for the difference may lie in the pH changes which alter with the flow rate. Cotinine has a pKa (dissociation constant) close to the pH of saliva and plasma. As the pH of unstimulated saliva is less than that of stimulated saliva, a basic compound such as cotinine would be influenced by the flow. It is possible to obtain maximum concentration of cotinine from saliva when the pH of saliva is acidic as in unstimulated saliva. Thus, as flow rate is increased with stimulation, less of the substance would be captured for measurement [6].

Unstimulated saliva was collected from all the patients. The patients were asked to rinse their mouth with water and seated comfortably with eyes open. They were asked to tilt their head slightly forward and rest for 5 minutes to minimize orofacial movements. Later they were asked to accumulate saliva in the floor of the mouth and repeatedly (every 60 seconds) spit in test tube through a funnel to collect ~5 mL of saliva. Then the saliva samples were refrigerated [12] at  $-20^{\circ}\text{C}$ . (Figure-2)

Then all the collected & stored samples were analysed for salivary cotinine levels by using Enzyme Linked Immunosorbant Assay (ELISA).

## Analysis of cotinine level's using ELISA

This kit (SHANGHAI YEHUA Biological Technology Co., Ltd, Cat. No: yhb0853Hu) [15] used Enzyme Linked Immunosorbant Assay (ELISA) based on Biotin double antibody sandwich technology to assay human salivary cotinine levels. The salivary samples stored at  $-20^{\circ}\text{C}$  were removed from refrigerator and allowed to settle down at room temperature and analyzed for cotinine. (Figure 3 to 10), (Table 1, 2)

**Inclusion criteria**

- Male patients with age group of 30-60 years.
- Smokers with chronic periodontitis.
- Non-smokers with chronic periodontitis.

**Exclusion criteria**

- Patients with any systemic diseases.
- Patients who were former smokers.
- Patients on systemic antibiotic therapy before 6 months.
- Patients consuming tobacco in any other form except cigarette smoking.
- Patient with history of previous periodontal treatment prior to study.

**RESULTS**

Level of significance was assessed at  $p \leq 0.05$ . For statistical analysis Mann-whitney U test and Spearman's rho ( $\rho$ ) were used.

**Intergroup comparison of variables (Table– 3)**

In this study age of the subjects ranged between 30-60years. For the statistical analysis age wise distribution of patients was done and they were divided into three sub groups. There was no statistically significant difference found in age of both the groups (Table 4). The median value of age in non-smokers and smokers were 42, 43 years respectively.

When Probing Pocket Depth was assessed between two groups, the median value for Group A was 4.3 and for Group B was 5.6 with  $p < 0.001$ . This shows that there is statistically significant difference was found between the groups. When Clinical Attachment Loss was assessed between two groups, the median value for Group A was 4.2 and for Group B was 5.3 with  $p < 0.001$ . This shows that there is statistically significant difference was found between the groups. When Plaque Index was assessed between two groups, the median value for both was 1.4 with  $p < 0.001$ . This shows that there is statistically significant difference was found between the groups. When Gingival Index

was assessed between two groups, the median value for Group A was 1.4 and for Group B was 0.86 with  $p < 0.001$ . This shows that there is statistically significant difference was found between the groups. When Gingival Bleeding Index was assessed between two groups, the median value for Group A was 43% and for Group B was 8% with  $p < 0.001$ . This shows that there is statistically significant difference was found between the groups. When Salivary cotinine levels were assessed between two groups, the median value for Group A was 4.65 pg/ml and for Group B was 42.31pg/ml with  $p < 0.001$ . This shows that there is statistically significant difference was found between the groups.

Cotinine levels and Fagerstrom index values were positively correlated in smokers with p value  $< 0.001$ . As the Fagerstrom value increases the salivary cotinine level was also increased.

**Correlation of clinical parameters with overall salivary cotinine levels**

Probing pocket depth, Clinical attachment level showed positive correlation with salivary cotinine levels. Gingival index, Gingival bleeding index showed negative correlation with salivary cotinine levels. Plaque index was not significantly correlated. (Table-5)

Overall Probing Pocket Depth values of both smokers and non-smokers were positively correlated with the overall salivary cotinine levels (p value  $< 0.001$ ). Overall Clinical attachment level values of both smokers and non-smokers were positively correlated with the overall salivary cotinine levels (p value  $< 0.001$ ). Overall Plaque index values of both smokers and non smokers were not significantly correlated with the overall salivary cotinine levels (p value of 0.623).

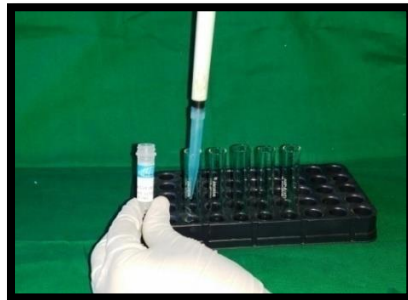
Overall gingival index scores were negatively correlated with overall salivary cotinine levels (p value  $< 0.001$ ). Overall gingival bleeding index were negatively correlated with overall salivary cotinine levels (p value  $< 0.001$ ).



**Fig-1: Collection of saliva**



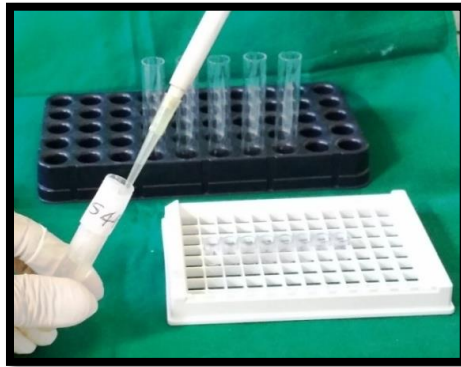
**Fig-2: -20°C Refrigerator**



**Fig-3: Dilution of standard solutions**



**Fig-4: Addition of 50µl of standard and 50µl of streptavidin-HRP to the standard solution well.**



**Fig-5:** Addition of 40 $\mu$ l of saliva sample to the sample well

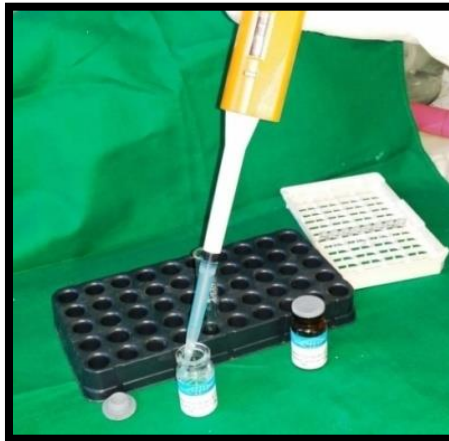


**Fig-6:** Wells incubated at 37 $^{\circ}$ C for 60minutes



**Fig-7:** Preparation of washing solution

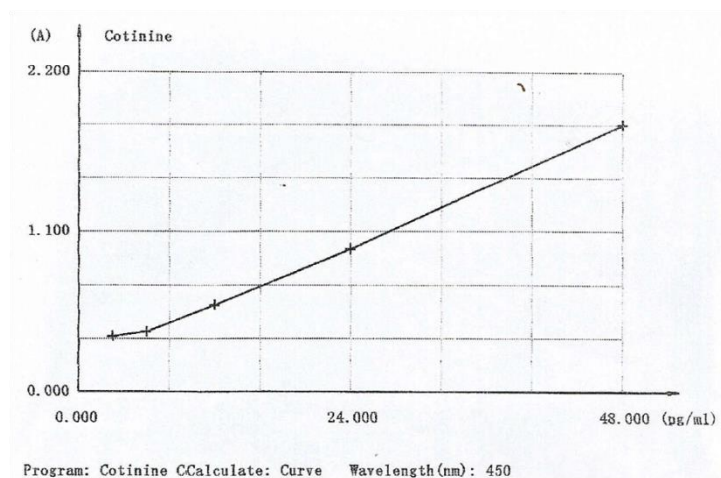




**Fig-8:** Addition of 50 $\mu$ l of chromogen reagent A, B to each well



**Fig-9:** Color change from blue to yellow



**Fig-10:** Standard curve

**Table-1: Dilution of standard solution**

48pg/ml	Standard No. 5	120µl original standard + 120µl of standard diluents
24pg/ml	Standard No. 4	120µl of standard No.5 + 120µl of standard diluents
12pg/ml	Standard No. 3	120µl of standard No.4 + 120µl of standard diluents
6pg/ml	Standard No. 2	120µl of standard No.3 + 120µl of standard diluents
3pg/ml	Standard No. 1	120µl of standard No.2 + 120µl of standard diluents

**Table-2: Standard table**

S. No.	Standards	Absorbance (450nm)	Concentration (pg/ml)
1	S1	0.380	3.00
2	S2	0.413	6.00
3	S3	0.592	12.00
4	S4	0.977	24.00
5	S5	1.834	48.00

**Table-3: Intergroup comparison of variables**

VARIABLES	NON- SMOKERS(Group A) <sup>a</sup>	SMOKERS(Group B) <sup>a</sup>	p-value <sup>b</sup>
AGE	42(31,59)	43(30,55)	0.692
PROBING POCKET DEPTH (PPD)	4.3(4.08,5.4)	5.6(4.4,7.4)	<0.001
CLINICAL ATTACHMENT LEVEL (CAL)	4.2(4.0,4.85)	5.3(4.2,7.2)	<0.001
PLAQUE INDEX (PI)	1.4(1.0,2.3)	1.4(0.4,2.5)	0.932
GINGIVAL INDEX (GI)	1.4(0.96,2.32)	0.86(0.12,1.4)	<0.001
GINGIVAL BLEEDING INDEX (GBI)%	43%(9%,78%)	8%(1%,25%)	<0.001
COTININE LEVEL (pg/ml)	4.65(1.26,18.12)	42.31(31.06,47.26)	<0.001

a: Median (Min, Max)

b: Mann-whitney U test was used to calculate the p-value

**Table-4: Age wise distribution**

Age Group	Smokers	Non-Smokers
<40	20(44.4%)	22(48.9%)
41-50	17(37.8%)	16(35.6%)
>50	8(17.8%)	7(15.6%)
<b>Total</b>	45	45

**Table-5: Correlation of overall clinical parameters with salivary cotinine**

CLINICAL PARAMETERS	COTININE LEVEL	
	SPEARMAN'S RHO (P)	P-VALUE <sup>a</sup>
PROBING POCKET DEPTH (PPD)	0.95	<0.001
CLINICAL ATTACHMENT LEVEL (CAL)	0.96	<0.001
PLAQUE INDEX (PI)	0.05	0.623
GINGIVAL INDEX (GI)	-0.94	<0.001
GINGIVAL BLEEDING INDEX % (GBI %)	-0.94	<0.001

A: Spearman's rho was used to find the p-value.

**DISCUSSION**

In 1997, World Health Organization (WHO) reported the prevalence of tobacco habits in India. It was found that 20% of population smoked cigarettes,

40% bidis and the remaining 40% is consumed chewing tobacco, pan masala, snuff, gutkha, masher and tobacco toothpaste [19]. It has been estimated that there were 1.1 billion smokers worldwide and 182 million

(16.6%) of them live in India [20]. Tobacco use kills more than 5 million people per year; this means 1 in every 10 adult deaths occur worldwide [21].

Chronic periodontitis is believed to be influenced by interaction of host defense and environmental factors. Potential causal association of cigarette smoking and periodontitis was studied by Gelsky *et al.* in 1999. He stated that smoking meets most of the criteria for causation proposed by Hill in 1965. This statement was based on the fulfillment of parameters between smoking and periodontal disease severity demonstrated by multiple cross-sectional as well as longitudinal studies [22]. An overwhelming body of data from multiple cross-sectional and longitudinal studies demonstrated that pocket depth and clinical attachment loss were more prevalent and severe in patients who smoke compared with non-smokers [4].

Jan Bergstrom *et al.* in 2000 [23] done a study to investigate the influence of smoking exposure over time on the periodontal health condition in a population before and after a follow-up interval of 10years. The results suggested that periodontal health is compromised by chronic smoking as evidenced by an increase of periodontally diseased sites concomitant with loss of periodontal bone height, as compared to non-smokers whose periodontal health condition remained unaltered throughout the 10year period of investigation.

In 2001 Haffajee and Socransky [24] from their study stated that increased prevalence of periodontal pathogens was caused by an increased colonization of shallow pockets and with no difference among smokers, former smokers and non-smokers in deep pockets. These data suggest that smokers have a greater extent of colonization by periodontal pathogens than non-smokers or former smokers and that this colonization may lead to an increased prevalence of periodontal breakdown.

The immune response of the host to plaque accumulation is essentially protective. In periodontal health and gingivitis, a balance exists between the bacterial challenge of plaque and the immune response from within the gingival tissues, with no resulting loss of periodontal support. The neutrophil is an important component of the host response to bacterial infection and alterations in neutrophil number or function may result in localized and systemic infections. Critical functions of neutrophils include chemotaxis (directed locomotion from the bloodstream to the site of infection), phagocytosis (internalization of foreign particles such as bacteria) and killing, using oxidative and non oxidative mechanisms [25]. In vitro studies of the effects of tobacco products on neutrophils have shown detrimental effects on cell movement as well as

the oxidative burst [26]. In addition, the production of antibody essential for phagocytosis and killing of bacteria, specifically, immunoglobulinG2 (IgG2) levels to periodontal pathogens, has been reported to be reduced in smokers versus non-smokers with periodontitis [26-29], suggesting that smokers may have reduced protection against periodontal infection. In contrast, elevated levels of tumor necrosis factor alpha (TNF- $\alpha$ ) have been demonstrated in the gingival crevicular fluid (GCF) of smokers, as well as elevated levels of prostaglandin, neutrophil elastase, and matrix metalloproteinase-8 (MMP-8) [30].

The oxygen concentration in healthy gingival tissues appears to be less in smokers than non-smokers, although this condition is reversed in the presence of moderate inflammation. Sub gingival temperatures are lower in smokers than non-smokers and recovery from the vasoconstriction caused by local anesthetic administration takes longer time in smokers.

Cigarette smoke contains more than 4,000 chemicals, including more than 60 carcinogens. Nicotine is the principal tobacco alkaloid, occurring to the extent of about 1.5% by weight in commercial cigarette and comprising about 95% of the total alkaloid content. On an average about 1–1.5 mg of nicotine is absorbed systemically during smoking<sup>6</sup>. The liver convert's nicotine to several metabolites and a small percentage, usually 5-10%, is excreted unchanged in the urine [7].

Nicotine is extensively metabolized to a number of metabolites by the liver. Six primary metabolites of nicotine have been identified. Quantitatively, the most important metabolite of nicotine in most mammalian species is the lactam derivative, cotinine. In humans, about 70–80% of nicotine is converted to cotinine. Six primary metabolites of cotinine have been reported in humans. 3'-Hydroxycotinine is the main nicotine metabolite detected in smokers. The metabolism of cotinine is much slower than that of nicotine. Cotinine clearance averages about 45 ml/min and that of nicotine is 1200ml/min [31].

Because of the long half-life of cotinine it has been used as a biomarker for daily intake, both in cigarette smokers and in those exposed to second hand tobacco smoke. There is, however, individual variability in the quantitative relationship between steady state cotinine levels and intake of nicotine. This is because different people metabolize cotinine differently at different rates (usual clearance range 20–75 ml/min) [31].

Martin *et al* from his study concluded that cotinine samples from blood, saliva and urine were



equally applicable to the whole range of issues requiring estimates of nicotine exposure from tobacco smoking. After cessation of smoking cotinine concentrations in all body fluids may be expected to decline to non-smoking levels within four days in the majority of cases, with an upper limit of seven days. Choice of fluid for sampling will depend on practical rather than pharmacokinetic considerations [8].

Cotinine level can be measured quantitatively using Enzyme Linked Immunosorbent Assay (ELISA) analysis, Gas chromatography, High Performance Liquid Chromatography (HPLC) and semi-quantitatively by reagent impregnated test strips. The cotinine levels found with ELISA were consistent with results from gas liquid chromatography [32].

Chamarthi Surya *et al.* [33] has done a study to assess a reliable marker of tobacco smoke exposure (salivary cotinine) by chair side reagent strip test to confirm the quantitative association between smoking and chronic periodontitis and concluded that quantitative direct association was established between salivary cotinine and severity of periodontitis.

In this study age of the subjects ranged between 30-60 years. For the statistical analysis age wise distribution of patients was done and they were divided into three sub groups. The analysis showed no statistically significant difference between two groups age wise. The median value of non-smokers and smokers were 42 and 43 years respectively. Only males were included in the study because there is high prevalence of cigarette smoking in males compared to females in India [34].

In this study Probing Pocket Depth (PPD), Clinical Attachment Levels (CAL) were calculated to measure the degree of periodontal destruction. Both PPD and CAL were significantly more in Group B when compared to Group A, in spite of the plaque scores showing no significant statistical difference. Similar findings have been reported in studies by, Surya *et al.* [33] and Sreedevi *et al.* [35]. This finding implies that the degree of periodontal destruction is more in smokers when compared to non-smokers.

Gingival index (GI) was higher in Group A than in Group B and the difference was found to be statistically significant. These findings were in consistence with the data Surya *et al.* [33].

Gingival bleeding index (GBI) was higher in Group than in Group B and the difference was found to be statistically significant. These findings were also in consistence with the data of Surya *et al.* [33]. Decreased gingival bleeding in smokers was explained as being due to nicotine, which causes vasoconstriction of

peripheral blood vessels such as in forearm, skin and hands.

In this study the median value of plaque index in Group A and Group B showed that there was no statistically significant difference between two groups. These results were in consistence with the findings of Sreedevi *et al.* [35]. This finding implies that the harmful effects of smoking on periodontal health may not be associated with plaque accumulation and poor oral hygiene.

In this study the median values of salivary cotinine levels for the intergroup comparison showed a statistically significant difference between the groups and it was significantly higher in Group B with p value <0.001. These findings were in agreement with the study by Yamamoto *et al.* [12]. This finding implies that cotinine levels in saliva were more in smokers than in non-smokers. The reason for the detection of salivary cotinine in non-smokers is because of exposure to environmental tobacco smoke.

The correlation between Fagerstrom index and salivary cotinine level in smokers was done which showed that a significantly positive correlation between them with p value <0.001. It indicates that with the increase in Fagerstrom value salivary cotinine levels also increased. The smokers who were depended more towards smoking have shown greater values of Fagerstrom index. These results were in agreement with the study by Heatherton *et al.* [16].

In this study the correlation between overall salivary cotinine values (i.e both in smokers and non-smokers) and overall clinical parameters showed a positive correlation for PPD, CAL and a negative correlation for GI, GBI. There was no significant correlation for PI. These results were in agreement with the studies by Gonzalez *et al.* [10]. This shows that as the salivary cotinine levels increased, the severity of periodontal parameters (i.e PPD, CAL) also increased. Tobacco smoke interacts with, and compounds the effects of various systemic conditions, resulting in greater disease severity. This finding also establishes a quantitative direct association between the level of tobacco smoke exposure and severity of periodontitis.

#### Limitations of the present study

Categorization of subjects as smokers and non-smokers may not be sufficient to evaluate the role of smoking in the severity of periodontal disease. This is because smokers represent a highly heterogeneous group of subjects. Instances where non-smokers can test positive for cotinine include environmental tobacco smoke (ETS) exposure. Individual metabolism, rate of absorption and ethnic differences also plays a role in the estimation of tobacco exposure which was not taken

into consideration. The sample included only males hence more number of studies including females also should be conducted.

## CONCLUSION

In this study the elevated cotinine levels in saliva showed a strong association for periodontal disease in smokers compared to non-smokers. The severe nicotine dependence also correlated positively to increased salivary cotinine levels. Hence quantitative assessment of cotinine levels can be considered as a strong diagnostic tool for assessing periodontal disease. Further longitudinal studies upon larger population are required to quantitatively assess the relationship between smoking and severity of periodontitis.

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