

Evaluation of salivary biomarkers of periodontitis among smokers and nonsmokers: A novel study

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ABSTRACT

Background: The analysis of salivary enzymes contributes to the clarification of pathogenesis and improvement in the diagnosis of periodontal disease. The present study aimed to examine the prospective association between smoking and periodontal disease progression and the effects of smoking on the following salivary biomarkers related to periodontitis: Alkaline phosphatase (ALP), acid phosphatase (ACP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), creatinine (CRE), blood urea nitrogen (BUN), urea (UA), free-hemoglobin (f-Hb), and neopterin. **Materials and Methods:** A total of 64 male patients aged 21–60 years were recruited and grouped as Group 1: 16 healthy nonsmokers, who had never smoked. Group 2: 16 smokers with gingivitis. Group 3: 16 smokers with moderate periodontitis. Group 4: 16 smokers with severe periodontitis. Stimulated saliva was collected for at least 5 min and clinical measurements; salivary biomarkers were assessed in automated analyzer. **Results:** Data showed significant correlation among salivary ACP, AST, LDH, CRE, BUN, UA, and f-Hb and neopterin levels showed higher in group 4 compared with other groups. **Conclusion:** This study indicated that smoking has several detrimental effects on periodontal tissues. A higher level of salivary biomarkers was seen in smokers with severe periodontitis. Hence, these biomarkers are helpful in future for the earlier detection of periodontal diseases progression and can also be used as potential salivary biomarkers for assessing smoking status and severity in chronic periodontitis.

Keywords: Biomarkers, chronic periodontitis, free-hemoglobin, neopterin, saliva, smoking

Introduction

Periodontitis is a chronic bacterial infection characterized by loss of connective tissue attachment and bone surrounding the teeth with the formation of periodontal pockets due to the apical migration of the junctional epithelium.^[1] Bacteria are the initiating agent in periodontitis, which along with the complexity of the associated microflora and the critical role of the host,

determines the outcome of the bacterial challenge causing difficulties in defining specific disease markers in periodontal diseases.^[1,2] The virulence factors released from the bacteria cause the direct degradation of host tissues and the release of biologic mediators from host tissue cells, which lead to tissue destruction. The mediators like proteinases, cytokines, and prostaglandins, which are produced as a part of the host response, also contribute to tissue destruction along with various bacteria-derived enzymes, such as collagen-degrading enzymes, elastase-like enzymes, trypsin-like proteases, aminopeptidases, and dipeptidyl peptidases, and other inflammatory mediators are recognized as important participants in tissue destruction which appear to hold

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Received: 23-10-2019

Revised: 30-12-2019

Accepted: 21-01-2020

Published: 28-02-2020

Access this article online

Quick Response Code:



Website:
www.jfmpc.com

DOI:
10.4103/jfmpc.jfmpc_937_19

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How to cite this article: Patil AS, Ranganath V, Kumar CN, Naik R, John AA, Pharande SB. Evaluation of salivary biomarkers of periodontitis among smokers and nonsmokers: A novel study. J Family Med Prim Care 2020;9:1136-42.

great promise as biomarkers for the diagnosis of periodontal disease.^[2,3] The analysis of the enzymes in salivary secretion and the gingival crevicular fluid contributes to the clarification of the pathogenesis along with improvement in making a prompt diagnosis of periodontitis. Those particularly related in this group of enzymes are the following: aspartate and alanine aminotransferases (AST and ALT), lactate dehydrogenase (LDH), creatine kinase (CK), alkaline phosphatase (ALP), acidic phosphatase (ACP), blood urea nitrogen (BUN), urea (UA), free-hemoglobin (f-Hb), and neopterin.^[4] ALP is a non-specific enzyme associated with the cell plasma membrane, which plays a role in the transport of substances from the intracellular compartment to the extracellular region. ALP is a good indicator of the metabolic activity of bone hence can be used as a possible biochemical marker in periodontal disease.^[5] ACP, a lysosomal marker, is present in neutrophils and is associated with bone metabolism. Due to the production of ACP by desquamated epithelial cells, macrophages, and several bacteria, its increased activity indicates a consequence of destructive processes in alveolar bone in advanced stages of the development of the periodontal disease.^[6] AST and ALT are cytoplasmic enzymes that catalyze amino-transfer or transamination, detected in periodontal tissue, GCF, the enamel pellicle, and saliva and are released after cell injury and cell death.^[7] LDH is a ubiquitous enzyme, catalyzes the reaction of lactate production via pyruvate reduction during anaerobic glycolysis.^[8] LDH release (leakage) is responsible for cellular membrane permeabilization and severe irreversible cell damage.^[9,10] End-stage renal disease has been shown to affect the oral and periodontal health of a patient along with the general health.^[10,11] CRE is a breakdown product of muscle tissue, which the kidney normally removes, is found both in saliva and plasma. Salivary levels of CRE share a close relationship with serum levels, with an average concentration ten times less than serum.^[12] UA is a final, organic compound of human catabolism of proteins. The hydrolysis of urea by bacterial urease enzymes generates ammonia and CO₂, which are potentially cytotoxic for the periodontal tissues.^[13] BUN represents the level of urea in the blood and inhibits nitric oxide synthesis and promotes macrophage proliferation.^[14] It indicates a higher level of cellular damage and reflects metabolic changes in the inflamed gingiva.^[15] F-Hb generates free hemoglobin complexes with LPS resulting in enhanced biological activity hence is considered as a potential biomarker.^[16] Neopterin is a metabolite of guanosine triphosphate (GTP) belonging to the class of pteridines, which is an early and valuable biomarker of cellular immunity. Neopterin takes part in nitric oxide synthase (NOS) production to produce nitric oxide (NO), which is a powerful antimicrobial substance produced in response to periodontal pathogens besides local inflammatory changes and the relationship between smoking and increased cardiovascular risk is well known. Cigarette smoking is a well-established risk factor for periodontitis and is associated with an increased risk for periodontal attachment loss (AL) and bone loss.^[17-19] Salivary analysis offers the potential to reflect current disease activity and severity, which can be advantageous for providing information used in yes/no decision matrices.^[7] To our knowledge, there are

no reports assessing ALP, ACP, AST, ALT, LDH, CRE, BUN, UA, f-Hb, F and neopterin activity in saliva and correlating their levels with periodontal disease in smokers and nonsmokers.

Hence, the purpose of the present study was to assess the levels of these salivary enzymes among healthy nonsmokers and smokers with periodontitis.

Materials and Methods

A total of 64 male patients aged 21–60 years were recruited from the Department of Periodontology, AECS Maaruti College of Dental Sciences and Research Centre, Bangalore. The design of the study was approved by the Ethical Committee, and all the patients were explained about the study and based upon their approval, were asked to read carefully and sign the informed consent form. Patients with systemic diseases that could influence periodontal conditions, including diabetes, immunity disorders and leukemia, periodontal treatment, or antibiotics or anti-inflammatory drugs taken within the previous 3 months, a history of regular use of mouthwashes, use of any vitamin supplementation or mucosal lesions, chemotherapy, radiation therapy or medications that cause xerostomia, and pregnancy and lactating females were excluded from the study. Patients were grouped as follows: Group 1: 16 patients who were nonsmokers with clinically healthy periodontium. Group 2: 16 patients who were smokers with gingivitis, i.e. when bleeding was observed after periodontal pocket probing. Group 3: 16 patients who were smokers with moderate periodontitis, i.e. when at least one probing depth was 4–5 mm, clinical attachment loss: 3–4 mm. Group 4: 16 patients who were smokers with severe periodontitis, i.e. when at least one probing depth was 6 mm or more, clinical attachment loss more than 5 mm. The definition of classification for moderate and severe periodontitis was according to the classification of the 1999 American Academy of Periodontology workshop.^[20] Assessment of smoking status was performed according to the criteria established by the Centre for Disease Control and Prevention (CDC) through a questionnaire. Patients who had smoked over 100 cigarettes over a lifetime and were smokers at the time of examination were included as smokers. Power analysis was calculated a sample size of 16 from a population of 100 has a 90% confidence interval with a probability of 0.05 and sum of the square of means equal to 1460889.79 when the standard deviation for the sample is 258.962. Therefore, the power of the study was calculated by using the above values and estimated to be 0.89. This infers that a sample size of 16 was adequate to get significant values when all the four groups were compared with each other.

Clinical parameters

Periodontal parameters such as gingival index (GI),^[21] probing pocket depth (PPD), and clinical attachment level (CAL) were measured at six sites and recorded on each tooth, except third molars. All the clinical parameters were performed using William's graduated periodontal probe by a single examiner.

Saliva sample collection and processing

All the patients were instructed to chew a piece of paraffin gum for 5 min to stimulate salivary flow, and 2 ml of stimulated saliva sample was collected in a sterile salivary vial between 9.00 am to 12.00 pm. Saliva samples were refrigerated at 4°C for ALP, ACP, AST, ALT, LDH, CRE, BUN, UA, and f-Hb and for analysis of neopterin were frozen at -80°C till further analysis. Salivary samples were analyzed to assess the following enzymes ALP, ACP, AST, ALT, LDH, CRE, BUN, and UA (Roche Diagnostics Pvt Ltd[®], India), which were analyzed by using Roche Modular P800-Hitachi Chemistry Analyzer; lactate dehydrogenase (DiaLab Ltd[®], Germany) was analyzed by using Tracer 30; f-Hb was measured by colorimetric method with reagents hydrogen peroxide (H₂O₂), 10% acetic acid, and ortho-toluidine; and neopterin kit (DRG Instruments GmbH[®], Germany) was analyzed by using ELISA. Absorbance was read at 450 nm and the results were calculated using the supplied formula or a data reduction system.

Statistical analysis

Analysis of variance (ANOVA) was used to find the significance of study parameters between the groups of patients. To set the cut-off points for the biochemical markers, receiver operating characteristic curves (ROC curves) were constructed and points showing the minimum difference between sensitivity and specificity were decided for gingivitis or periodontitis. The Chi-square test was carried out to confirm the statistical significance, and positive or negative predictive values were calculated for the biochemical markers. *P* value ≤ 0.05 was considered as statistically significant.

Results

Table 1: The mean levels of AST, ACP, CRE, LDH, CRE, BUN, UA, fHb, LDH, and neopterin were higher, whereas ALP and ALT levels were lower in group 4 when compared group 1, 2, and 3. An overall *P* value of <0.05 was obtained for all the parameters, suggesting a statistical significance in all the four groups included in the study. The levels of salivary enzymes ALP were lowest for the group 4 when compared group 1, 2, and 3. The mean levels of ALT were higher in group 1 and lower in group 3. Table 2: The mean values for periodontal parameters GI, PPD, and CAL were higher in group 4 compared to group 1, 2, and 3. Table 3: Cut-off point, sensitivity, specificity, and area of ROC (receiver operating characteristic) curves were calculated using Chi-square test. ALP and ALT showed statistically insignificant values. For Sensitivity: LDH (100) and CRE (100) showed maximum values, followed by ACP (93.75), f-Hb (93.75), neopterin (93.75), AST (87.50), UA (75), and BUN.

For specificity

BUN (95.83) showed highest values followed by f-Hb (89.58), LDH (89.58), ACP (85.42), UA (85.42), neopterin (85.42), AST (79.17), and CRE (52.08).

LDH and CRE have maximum sensitivity and BUN and f-Hb have maximum specificity. Table 4: Area under curve (AUC) values in decreasing order were LDH (0.99), f-Hb (0.96), neopterin (0.95), UA (0.91), ACP (0.86), AST (0.86), BUN (0.82), and CRE (0.77). Figure 1 infers that LDH has maximum better overall performance as a diagnostic test followed by f-Hb, neopterin, UA, ACP, AST, BUN, and CRE.

Discussion

Gingivitis and periodontitis are the most prevalent chronic inflammatory conditions that affect as much as 80% of the adult population.^[22] The activation of patient's host response liberates a myriad of metabolic byproducts at the interface between the tooth and the periodontal pocket which leads to the release of destructive cellular enzymes, cytokines, chemokines, and other mediators of tissue destruction.^[23] There are numerous markers in saliva which have been proposed and used as diagnostic tests for periodontal disease but the diagnostic tests should demonstrate sensitivity and specificity. It is improbable that a single marker will prove to be both sensitive and specific due to the complexity of the periodontal disease. A combination of two or more markers can provide an accurate assessment of the periodontal patient. To fulfill this, numerous biomarkers and diagnostic tests were developed, demonstrating high levels of sensitivity, specificity, and diagnostic accuracy with respect to identifying and/or predicting disease activity at the site level.^[24] Alkaline phosphatase and acid phosphatase are intracellular enzymes present particularly in bones.^[4] ALP is enriched in membranes of mineralizing tissue cells, polymorphonuclear leukocytes (PMN) granules and produced by gram-negative microorganisms found in subgingival plaque.^[25] For ALP, our results are in agreement with the study done by Nomura *et al.*^[26,27] which showed statistically insignificant levels in saliva of smokers with periodontitis. However, studies done by Todorovic *et al.*,^[4] Totan *et al.*,^[5] and Dabra *et al.*^[25] showed statistically significant

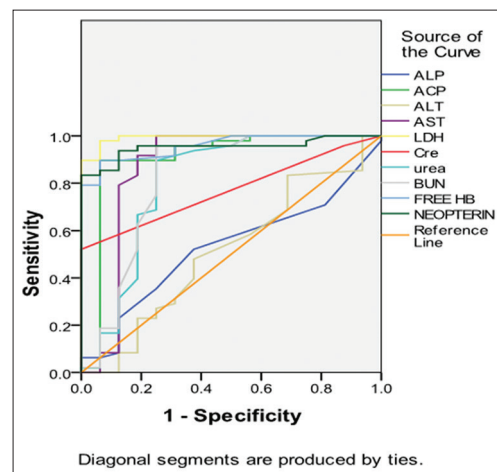


Figure 1: ROC (Receiver Operating Characteristic) curves of biochemical markers tested for screening of periodontitis Alkaline Phosphatase: ALP, Aspartate Aminotransferase: AST, Acid Phosphatase: ACP, Alanine Aminotransferase: ALT, Blood Urea Nitrogen: BUN, Free-Hemoglobin: Free-Hb, Lactate Dehydrogenase: LDH

Table 1: Comparison of salivary levels of various biochemical markers among subjects without periodontal disease, subjects with gingivitis, subjects with moderate periodontitis, and subjects with severe periodontitis

	No periodontal disease (n=16)		Gingivitis (n=16)		Moderate periodontitis (n=16)		Severe periodontitis (n=16)		P
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
ALP (U/L)	3.375	3.3441	5.500	4.3205	3.563	6.0439	2.438	1.3150	0.210
AST (U/L)	25.125	30.2123	36.688	13.3002	38.125	15.1388	48.688	11.0858	*0.010
ACP (U/L)	19.869	15.5565	39.956	10.7186	39.594	8.9121	48.881	13.4903	*0.000
ALT (U/L)	14.563	21.4941	12.688	11.4001	6.688	8.0971	11.125	10.7012	0.429
CRE (U/L)	0.113	0.0342	0.125	0.0577	0.163	0.0500	0.169	0.0602	*0.000
BUN (U/L)	11.313	7.0401	15.375	5.2520	15.313	3.2806	21.000	10.7455	*0.004
UA (U/L)	24.375	15.1256	28.125	6.3127	33.875	5.8751	45.063	21.9347	*0.001
FREE-HB (mg/dL)	0.2625	0.17650	0.9988	0.45639	0.9138	0.38631	1.3200	0.23267	*0.000
LDH (U/L)	62.19	21.858	153.25	23.530	138.80	21.637	330.69	91.490	*0.000
Neopterin (ng/ml)	1.06263	0.12234	1.06344	0.15507	1.10063	0.13483	1.18675	0.353491	*0.007

*P is statistically significant at 0.05. P were calculated using one way ANOVA. Alkaline Phosphatase (ALP), Aspartate Aminotransferase (AST), Acid Phosphatase (ACP), Alanine Aminotransferase (ALT), Creatinine (CRE), Blood Urea Nitrogen (BUN), Urea (UA), Free-Hemoglobin (Free-Hb), Lactate Dehydrogenase (LDH), Neopterin

Table 2: Mean values of gingival index (GI), probing pocket depth (PPD), and clinical attachment level (CAL) in all four groups

	No periodontal disease (n=16)		Gingivitis (n=16)		Moderate Periodontitis (n=16)		Severe Periodontitis (n=16)		P
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
GI	0.0856	0.02394	0.0513	0.01628	1.3163	0.08302	1.6644	0.17340	*0.000
PPD (mm)	1.8975	0.18325	1.9363	0.15790	3.2488	0.59408	3.8844	0.40989	*0.000
CAL (mm)	1.8975	0.18325	1.9363	0.15790	3.3300	0.57372	4.0831	0.40143	*0.000

*P is statistically significant at 0.05. P calculated by using one way ANOVA. Gingival Index (GI), Probing Pocket Depth (PPD), Clinical Attachment Level (CAL)

Table 3: Cut-off point, sensitivity, and specificity of various biochemical markers in saliva

Biochemical markers	Cut-off point	Gingivitis or periodontitis		Chi-square	*P	Sensitivity	Specificity	NPV	PPV	
		Present	Absent							
		ALP (U/L)	2.50							<2.50 ≥2.50
ACP (U/L)	30.50	<30.50 ≥30.50	15 1	5 43	38.79	<0.0001	93.75	89.58	97.73	75
ALT (U/L)	5.50	<5.50 ≥5.50	7 9	20 28	0.02	>0.05	43.75	58.33	75.68	25.93
AST (U/L)	28.50	<28.50 ≥28.50	14 2	10 38	22.76	<0.0001	87.50	79.17	95	58.33
LDH (U/L)	125.50	<125.50 ≥125.50	16 0	5 43	43.68	<0.0001	100	89.58	100	76.19
Creatinine (U/L)	0.15	<0.15 ≥0.15	16 0	23 25	13.67	<0.0001	100	52.08	100	41.02
Urea (U/L)	22.50	<22.50 ≥22.50	12 4	5 43	25.66	<0.0001	75	89.58	91.49	70.59
BUN (U/L)	10.50	<10.50 ≥10.50	12 4	2 46	35.23	<0.0001	75	95.83	92	85.71
FREE HB (mg/dL)	0.465	<0.465 ≥0.465	15 1	5 43	38.79	<0.0001	93.75	89.58	97.73	75
NEOPTERIN (ng/mL)	1.26	<1.26 ≥1.26	15 1	7 41	33.34	<0.0001	93.75	85.42	97.62	68.18

*P-values calculated by Chi-square test. Alkaline Phosphatase : ALP, Aspartate Aminotransferase : AST, Acid Phosphatase : ACP, Alanine Aminotransferase : ALT, Blood Urea Nitrogen : BUN, Free-Hemoglobin : Free-Hb, Lactate Dehydrogenase : LDH, PPV: Positive Predictive Value, NPV: Negative Predictive Value

results in patients with periodontitis compared with controls. Kibayashi *et al.*^[28] indicated decrease in levels of ALP suggesting that impairment of an inflammatory response in the development of periodontitis as a consequence of current smoke exposure. Todorovic *et al.*^[4] and Dabra *et al.*^[25] showed increase in levels of ACP levels in patients with periodontitis which is in agreement

with our study. LDH activity has been studied extensively in various tissues and plasma but studies in saliva are scarce. Within the cells, glucose is used principally for the production of pyruvate in the glycolysis pathway. In aerobic conditions, pyruvate enters the mitochondrial matrix, undergoes oxidation by the action of pyruvate dehydrogenase and transforms into acetyl-CoA which

Table 4: Values showing area under curve (AUC)

Biochemical markers	AUC	Significance	95% CI	
ALP	0.53	0.751	0.37	0.68
ACP	0.91	0.000	0	1
ALT	0.51	0.889	0.34	0.68
AST	0.86	0.000	0.67	1
LDH	0.99	0.000	0	1
Creatinine	0.77	0.001	0.66	0.88
Urea	0.81	0.000	0.65	0.97
BUN	0.82	0.000	0.67	0.98
FREE HB	0.96	0.000	0.91	1
NEOPTERIN	0.95	0.000	0.90	1

CI: Confidence Interval. Alkaline Phosphatase: ALP, Aspartate Aminotransferase: AST, Acid Phosphatase: ACP, Alanine Aminotransferase: ALT, Blood Urea Nitrogen: BUN, Free-Hemoglobin: Free-HB, Lactate Dehydrogenase: LDH

enters the citric acid cycle.^[9] In anaerobic conditions, pyruvate is reduced to lactate in a reversible reaction catalyzed by LDH, where nicotinamide adenine dinucleotide is used as coenzyme. LDH activity provides information on cellular glycolytic capacity. Therefore, to study and characterize salivary LDH as a potential diagnostic tool is of great interest to modern medicine.^[9] AST and ALT are cytoplasmic enzymes which serve as diagnostic analytes of cellular injury and are relevant to periodontal disease diagnosis. Interestingly, substantially higher levels of AST and ALT showed higher levels in the saliva of healthy subjects as compared with serum as their levels in periodontitis relate to the type of tissue affected by necrosis.^[7]

The results of the present study showed significant increase in levels of AST and LDH in smokers with periodontitis as compared with controls; however, ALT showed significant decrease in its levels. This was in agreement in study done by Nomura *et al.*,^[26] which showed similar results. In a study by Rai *et al.*,^[29] salivary levels of ALT, AST, and LDH showed significantly higher levels in smokers with severe periodontitis as compared to controls since total antioxidant activity has been reported to be decreased in saliva of patients with periodontitis as compared to those with healthy periodontium. Mohammad^[9] concluded a positive correlation in salivary levels of ALT, AST, and LDH in smokers with periodontitis. The alteration in host bacterial interaction associated with smoking leads to a more aggressive breakdown than which is normally seen in chronic periodontitis. The imbalance between bacterial challenge and host response is due to changes in composition of the subgingival plaque with an increase in the numbers and/or virulence of the pathogenic organisms, which changes the host response to the bacterial challenges or combination of both.^[9,30,31] The chronic effect of smoking on salivary AST remains unclear but the AST levels in saliva were correlated with periodontal diseases evaluated with the community periodontal index, which suggests that periodontal destruction such as periodontal pockets, gingival bleeding, and suppuration may be related to higher AST levels in saliva.^[31] Results of a study done by Kibayashi *et al.*^[28] showed significantly lower levels of AST and LDH were lower in current smokers than in

non-current smokers. Activities of AST and LDH in saliva were inhibited after smoking of a single cigarette and this was attributed to the interaction between aldehydes within smoke and the thiol (-SH) groups of enzyme molecules. In an *in vitro* study by Avezov *et al.*^[32] cigarette smoke caused a reduction in LDH activity, in which α,β -unsaturated aldehydes, main cigarette smoke ingredient, were responsible for salivary LDH activity diminution as they introduce carbonyl group into proteins causing their dysfunction.^[32] Extensive literature exists for the relationship between periodontal disease and cardiovascular diseases. Many studies provided evidence for an increased prevalence of periodontal disease in patients with renal disease, especially in dialysis patients and renal transplant recipients.^[11] Chronic renal failure is a slow, progressive loss of renal function over months or year which shares many common risk factors with atherosclerotic cardiovascular disease, imbalances in bone metabolism, and chronic kidney disease. It begins without symptoms initially and as renal function decreases, blood pressure increases, urea accumulates, leading to uremia and fluid volume overload.^[33] CRE is found in both saliva and plasma; hence, it is a key diagnostic enzyme for kidney disease. The kidney produces urine mainly through passive diffusion, which is the main mechanism at work in the formation of saliva. Constituents leaving the blood in the kidneys should be similar to those leaving the blood at the salivary gland. Because of this similarity, a creatinine clearance test performed on saliva provides the same information in an easier and more efficient manner.^[10,12]

Many studies have reported biochemical changes in the saliva of patients undergoing dialysis within relationship to urea.^[11] The hydrolysis of urea by bacterial urease enzymes generates ammonia and CO₂ which is considered a significant pathway for alkali production in the oral cavity. Ammonia, which is potentially cytotoxic, increases the permeability of the sulcular epithelium to other antigenic and toxic substances and plays a fundamental role in the initiation of gingivitis.^[13] Blood urea nitrogen has pro-atherosclerotic effects, as uremia has been associated with an increased burden of oxidative stress. Elevated BUN serves as a marker of an activated sympathetic nervous system and/or an upregulated renin-angiotensin system which are promoters of atherosclerosis; the activation of these neurohormonal systems is associated with increased BUN reabsorption in the renal tubules.^[14]

The results of the present study show statistically significant increase in levels of UA, CRE, and BUN in smokers with severe periodontitis as compared with control groups. The results of the present study were in agreement with that of Nomura *et al.*^[26] which showed increased levels of these enzymes. In a study done Bezerra *et al.*^[13] and Mahmood^[15] which also showed increase in urea levels in chronic periodontitis as increase in urea is associated with bacterial urease activity, especially from gram-negative anaerobic bacteria, and the consequent formation of ammonia, which is cytotoxic for the periodontal tissues. These enzymes are an indication of a higher level of cellular damage and their

increased activity is a consequence of their increased release from the damaged cell, which reflects metabolic changes in inflamed gingival.^[15] Neopterin is an actual molecular mass compound known to take part of a process of NO production which may be part of the non-specific natural defense mechanisms of the oral cavity against periodontal pathogenic bacteria.^[16]

The results of the present study show statistically significant increase in levels of neopterin in smokers with periodontitis as compared to control group. Studies by Pradeep *et al.*^[34] and Ozmeriç *et al.*^[16] show increase in neopterin levels in patients with chronic periodontitis. Increased neopterin concentrations indicate cell-mediated immune activation and enhance the cytotoxic potential of activated macrophages and dendritic cells through the activation of reactive oxygen species (ROS).^[34] F-Hb is shown to be statistically increased in smokers with periodontitis. The results are in agreement with studies done by Nomura *et al.*^[15] Ferrrous iron has been established to catalyze the generation of radicals, which can modify lipids, proteins, and DNA, which induces inflammation. The development of microbes is critically reliant on iron, which might serve as additional explanation for the detrimental effects of hemolysis. Hemolysis can also result in the generation of micro particles, which are able to induce inflammation and disseminated intravascular coagulation.^[35] According to our knowledge, there are no reports of a previous study where area under the curve values were calculated for ALP, ACP, AST, ALT, LDH, CRE, BUN, UA, f-Hb, and neopterin. The results of the present study showed LDH has maximum area under the curve. Therefore, LDH has maximum better overall performance as a diagnostic test followed by Free-Hb, neopterin, UA, ACP, AST, BUN, and CRE.

According to the results of present study, group 4 showed higher values of periodontal parameters values as compared to the group 1, 2, and 3. This was in agreement with the studies done by Bergstrom and Bostrom^[36] and Dietrich *et al.*^[37] Smoking is identified to produce peripheral vasoconstriction, in some subjects, this is regulated by vasodilatation. In any particular instance, the effect produced is perhaps associated to the degree of inhalation of the tobacco smoke and the rate of nicotine concentration. Nicotine from cigarette causes the stimulation of the sympathetic ganglia to produce neurotransmitters including catecholamines. The alpha-receptors on blood vessels are affected by catecholamines, which cause vasoconstriction of peripheral blood vessels caused by smoking and also affect the periodontal tissue. Smokers have less overt signs of inflammation including clinical signs such as redness, bleeding, and exudation.^[38] The vasoconstrictive actions of nicotine cause the decreased gingival blood flow.^[37] The results of our study showed increased probing depths and clinical attachment loss in smokers with periodontitis as compared to the healthy group.

This is in agreement with studies done by Calsina,^[39] Torrungruang *et al.*,^[40] Martinez-Canut P,^[41] Gunsolley,^[42] and Susin,^[42] who concluded that smokers exhibit elevated total white blood cell and granulocyte counts in their systemic

circulation; however, the influence of cigarette smoking on PMN cell numbers in the gingival crevice is not clear. Cigarette components alter the PMN viability and functions including phagocytosis, superoxide and hydrogen peroxide generation, integrin expression, and protease inhibitor production. PMN demonstrate more destructive activities leading to increased periodontal destruction.^[40-42]

Conclusion

Tobacco smoking is one of the common causes of oral cancer in India. Preventive measures should be carried out in primary health care centers by behavioral counseling interventions, such as face-to-face or telephone interaction with a health care professional, computer applications, educational videos, printed materials, and assessing the salivary biomarkers, which can reduce the risk of smoking initiation. Hence, within the limitations of the study, ACP, AST, LDH, CRE, BUN, UA, f-Hb, and neopterin levels showed statistically significant increase in smokers with severe periodontitis. LDH and CRE have maximum sensitivity and BUN and f-Hb have maximum specificity. Based on area under curve values, LDH showed maximum better overall performance as a diagnostic test. These markers can be significant diagnostic implement as salivary biomarkers in smokers with periodontal disease.

Financial support and sponsorship

This study was supported by the Indian Council of Medical Research (ICMR), New Delhi.

Conflicts of interest

There are no conflicts of interest.

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