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Diagnostic accuracy of salivary hemoglobin, lactate dehydrogenase and Interleukin-6 to determine chronic periodontitis and tooth loss in type 2 diabetics

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ABSTRACT

Background: Salivary Hemoglobin (SH) has emerged as the mainstay non-invasive and a practicable screening method for Chronic Periodontitis. Current research aims to comprehensively assess the diagnostic value of Salivary Hb (SH) in comparison with Salivary IL-6 (SIL-6) and levels of Salivary lactate dehydrogenase enzyme (SLDH) amongst Type II Diabetes subjects having Chronic Periodontitis (CP) and associated tooth loss.

Materials and methods: In this cross-sectional comparative investigation, 240 individuals with at least 15 remaining teeth, ranging in age from 30 to 70, were chosen and Group I controls were defined as follows: healthy (HbA1c levels $\leq$6.4 %) with no CP; Group II included chronic periodontitis and non-T2DM (HbA1c $\leq$ 6.4 %); Group III included T2DM (HbA1c $\geq$ 6.5 %) and CP; and Group IV included T2DM (HbA1c $\geq$ 6.5 %) with periodontitis-related tooth loss. ELISA colorimetric assay was used to quantify the results using the unstimulated whole saliva of fasting participants. Tukey's post hoc test was used for statistical analysis following Analysis of Variance (ANOVA), and Sensitivity and Specificity were computed following the determination of the correlation coefficient.

Results: One-way ANOVA comparing Biomarker levels across the four groups revealed a statistically significant difference ($F = 68.013$) ($p = 0.0001$). Tukey's multiple post hoc yielded a significant difference between groups with least mean average biomarker levels observed among the controls (Group1) and maximum with group IV. Diagnostic Accuracy to discriminate between CP in T2DM & Controls with SH surpassed that of SIL-6 & SLDH, Receiver operating characteristic (ROC) curve depicted an overall sensitivity of 67.62 %, specificity of 80 % and accuracy of 74 % in T2DM subjects with tooth loss for the identification and assessment of CP.

Conclusion: Estimates of Salivary Hemoglobin can assume an important role in comparison to SIL-6 & SLDH in determining the degree of periodontitis, including tooth loss, and identifying elevated glycemic levels. Advanced detection and monitoring can be ensured by routine use in dental offices and general practice.

1. Introduction

It is discovered that oral microbial dysbiosis and mostly increased inflammatory mediators in serum and saliva are present in T2DM patients, and these factors contribute to the etiology of periodontitis.[1] Despite the disease burden being ubiquitous, dental health guidelines are not given specific focus in the American Diabetes Association's Standards of Medical Care in Diabetes.[2] Globally lack of understanding and prioritising dental health amongst diabetics regardless of its severity is ascertained as an important causative factors in tooth loss, which is the clear result of the advancement of periodontal disease.[3]

Hyperglycemia creates an excessive amount of advanced glycation

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end products (AGEs) and osteoblasts, which produce downstream effectors like receptor activator of nuclear factor-kappa B ligand (RANKL), with an expression on osteoclasts and contributing to the destruction of alveolar bone, in an irreversible manner.[4] It has been determined that intracellular enzymes, such as LDH, alanine aminotransferase, and aspartate aminotransferase, secreted into saliva and crevicular fluid of gingiva by periodontal tissue-damaged cells and Interleukin 6 (IL-6) abundantly secreted by the fibroblasts of the gingival and periodontal ligament as a reaction to an infection with bacteria are clinically valuable indicators of tissue death and inflammation.[5,6]

Chronic periodontitis can result in the breakdown of the epithelial barrier, which opens the door for blood and its constituents to seep into saliva. Free hemin promotes the growth of gingivitis-causing bacteria and is present in cells at very small concentrations (<1 μM), is produced by red blood cell breakdown or vascular injury.[7] Recently, the SH levels has been assessed as a periodontitis marker, and their monitoring helps to avoid tooth loss brought on by periodontal disease,[8,9] pilot tested by authors in type 2 Diabetics and periodontal disease,[10] and the Pharmaceutical Affairs Law for Extracorporeal Diagnostic Agents in Japan, which authorizes the use of hemoglobin levels present in saliva to evaluate periodontal diseases, has further supported this conclusion.[11]

Literature has discovered factors that contribute to bidirectional relationship with Type 2 DM - periodontal disease referring to the pathogenesis, therapy, and their prophylaxis.[12] Further, with the limitations of conventional periodontal examination by the dental professional, a reliable and affordable non-invasive screening method technique for evaluating the periodontal health of large population groups is the need of the hour. With studies globally affirming Salivary hemoglobin, Salivary LDH and Salivary Interleukin −6 as screening tools for periodontitis. Thus, we aimed in this study to evaluate their performance with the following research question. Do subjects with clinically diagnosed chronic periodontitis, Type 2 diabetes and with tooth loss are correlated with diagnostic levels of Salivary Hb, IL-6 and LDH in whole unstimulated saliva than those in the control?

2. Materials and methods

Current double-blind study was approved under IEC Research Protocol No.: 15/2020, aligned with 1975 Helsinki Declaration (revised in 2000). An estimated 240 participants were needed to attain 80 % power and alpha error of 5 % in the sample. Volunteered participants were aged between thirty and sixty-nine, have at least fifteen natural teeth left, be free of dental implants, restorations, caries, mucosal diseases, oral pathologies, and have visited the outpatient department (OPD) at least once or frequently, have provided informed consent, meeting the criteria of inclusion and exclusion.

2.1. Clinical examination

A dentist performed oral examination, recorded demographic information, and interviewed subjects for their medical and dental history. Those who had appointments for forthcoming treatments or procedures were receiving medical or dental care from a healthcare professional in the month prior to the screening appointment also eliminated. Intra-oral examination performed at the Dental Out-patient Department,oral hygiene index—simplified was evaluated and performed by a single examiner (OHI-S). Mesio-facial, facial, disto-facial, mesio-lingual, lingual, and disto-lingual were the six sites per tooth that were probed with a PCP-UNC-15 probe. The greatest probing depth and largest clinical attachment loss (CAL) of each tooth were recorded. Centers for Disease Control and Prevention-American Academy of Periodontology (CDC-AAP) 2003 categorization of no periodontitis, mild, moderate, and severe periodontitis was involved to further categorize the subjects.[13] Furthermore, the average clinical attachment loss (APPD) was calculated by dividing the total number of teeth with a pocket depth greater than 4 mm by the greatest probing depths of all teeth.[14]

2.2. Subject stratification

Enrolled subjects were divided into the four groups listed below based on the results of the baseline assessment. Healthy (Group 1): HbA1c levels ≤6.4 %[2] without CP; good oral hygiene, less than 3 mm of probing depth, and no clinical attachment loss, no history of type 2 diabetes, no systemic medication therapy at that time; Chronic Periodontitis (Group 2): Healthy individuals with HbA1c levels ≤6.4 %[2] with chronic periodontitis (CP);[13,15] Type 2 DM with Chronic Periodontitis (Group 3): HbA1c values ≥ 6.5 % beyond two years and with CP; Type 2DM with Tooth loss (Group 4): Individuals with periodontitis-related tooth loss, subjects with history of periodontitis-related tooth loss or recommended for extraction due to periodontitis and HbA1c levels ≥6.5 %.

Exclusion criteria had subjects with inability to communicate, trouble gathering saliva, oral mucosal illness or wounds that cause oral bleeding and treatment for periodontal disease lesser than four weeks before saliva sampling. Subjects with a past medical history of systemic disorders, alcohol abuse, tobacco use, neurological illness, endocrine and metabolic problems impacting serum/salivary glucose levels, except for type 2 diabetes mellitus, salivary gland surgical inteventions, autoimmune conditions, chemotherapy, receiving long-term local and systemic drug therapy, antibiotics, and anti-inflammatory drugs (except oral hypoglycemics), pregnant subjects and not ambulatory.

2.3. Saliva sampling

After an 8-h fast, between 8 and 10 a.m., unstimulated saliva (about 5 ml) was collected after 30 s of rinsing with 10 mL of drinking water. Wide-mouth disposable sterile tubes with distinct subject identification (adapted Navazesh technique)[16] were utilized, and saliva samples were immediately placed on ice, transferred for biochemical analysis without delay, separated into aliquots, and kept in ideal storage conditions (long-term storage at 80 °C; short-term storage at 20 °C).

2.4. Quantification of Saliva sample for free hemoglobin, salivary IL-6 and salivary LDH levels

Utilizing a commercially available hemin colorimetric assay kit (Catalog #K672-100) BioVision Inc CA, salivary heme was quantitatively quantified. The saliva sample was ascertained for quantity of free hemoglobin in the samples by spectrophotometrically measuring its optical density (OD) using the PerkinElmer Enspire Multimode Plate Reader at 570 nm following a Coupled Enzyme reaction. Fixed on the idea that hemin functions as a peroxidase in samples, the assay measures the transformation of a colorless probe into a brightly colored compound using a spectrophotometer.

Using a lactate dehydrogenase activity test kit that is sold commercially (Sigma Aldrich, USA), saliva samples were subjected to a colorimetric assay to measure the amount of the oxidoreductase enzyme lactate dehydrogenase (LDH) in IU/L. The optical density at 450 nm was measured by spectrophotometry using the Tecan Infinite M200 Pro multiwell microplate reader as part of the catalytic reaction, which entails the LDH enzyme reducing NAD to NADH.

Salivary levels of IL-6 were assessed in pg/mL with the commercially available Human Interleukin-6 ELISA Kit (Sigma Aldrich, USA) as prescribed by the manufacturer using Sandwich ELISA technique. The Tecan Infinite M200 Pro multiwell microplate reader was used to measure the optical density at 450 nm using spectrophotometry. Readings of the optical density were utilized to plot standard curve and extrapolate the salivary hemoglobin LDH and IL-6 biomarker levels.

2.5. HbA1c measurement

Using the High-Performance Liquid Chromatography (HPLC) method, the HbA1c of each patient was estimated and reported as a

percentage. When the HbA1c level was 6.4 %, all subjects—including prediabetics—were classified as non-diabetic; and 6.5 %, - classified as diabetic.[2]

Study ensured blinding across the study process as the samples were coded and the study was carried out by a single examiner (senior research fellow, ICMR) after calibration to minimize bias during data collection and analysis.

2.6. Statistical analysis

The data was entered into an Excel spreadsheet and then analysed using the statistical tool SPSS Version 20.0.Two-sided statistical tests were conducted to assess the hypotheses, with statistically significant findings reported at 5 % ($p < 0.05$). The data was subjected to parametric testing. Tukey's multiple comparison tests were used to determine group differences, and Analysis of Variance (ANOVA) was used to compare the results from each group followed by Karl Pearson's correlation coefficient to assess the association. The results of clinical evaluations of Chronic Periodontitis across groups and the demographic features of recruited participants calculated from each clinical group were reported using descriptive statistics.

3. Results

The study's participants were categorised on the basis of methodology described; were then split up into four groups, with 60 individuals each, total of 240 participants, as depicted in Table 1. The study subjects comprised of 104 males and 136 females having a statistically significant gender distribution across each group. Overall mean age was 48.86 ± 6.90. Analysis of mean ages across groups with ANOVA followed by Tukey's multiple posthoc procedures suggesting that the mean age of subjects in Group 4 (T2DM with CP & Tooth Loss) were higher than controls significantly.

Comparison of four groups for dental history and oral hygiene parameters Table 2 we found no statistically significant difference of history of dental check-ups, last oral prophylaxis, use of interdental cleaning aids, number of times teeth cleaned per day and presence of prostheses across the groups. Although, history of tooth loss due to periodontitis was found statistically significant. The results presented in Table 2 demonstrates no statistically significant difference in oral hygiene index – simplified inference among the four groups ($p = 0.054$), but gingival index score increased significantly among the four groups ($p = 0.0001$).

The average SH, SIL-6, and SLDH biomarker levels were compared using a one-way ANOVA, and the results showed a statistically significant difference between the four groups. Salivary Hb ($F = 23.453$) ($p = 0.0001$) was significantly greater than SIL-6 and SLDH. Tukey's multiple post hoc applied for pairwise comparison among the four groups. This yielded a significant difference between group I vs group IV, group II vs group IV, and group III vs group IV. No discernible change between groups I vs II, and groups I vs III suggesting that Group I had least mean average biomarker levels as compared to maximum with Group IV (Table 3, Fig. 1).

The direction and degree of relationship of average periodontal pocket depth with biomarker levels were calculated using Karl Pearson's correlation coefficient, which showed positive correlation ($r = 0.4224$, $p < 0.05$) compared to SIL-6 and SLDH, according to Table 4 inferring that SH and average pocket depth are interdependent even though the points are spaced out over a wider band as depicted in the scatter plot diagram (Fig. 1). The category of severe periodontitis had significantly higher mean average biomarker values than the mild and moderate categories. All the Biomarkers noticeably had significant difference in between no periodontitis to severe periodontitis. As demonstrated in Table 5 depicting distribution of subjects across severity of Periodontitis 26 (43.3 %),15 (25 %)

40 (66.7 %) of Group 2, 3,4 respectively had severe Periodontitis. Results of one-way ANOVA comparing the four groups' levels of periodontitis severity found a statistically significant difference, with a larger F-value for SH ($F = 22.9334$) ($p = 0.0001$) than for SIL6 & SLDH, indicating a significant variation in the group means. The results of the post hoc analysis showed that group I (controls) had the lowest mean average biomarker levels, whereas group IV had the highest as in Table 6.

A biomarker's primary attributes are its clinical and analytical performance. Logistic Regression analysis was selected to determine the tests' clinical application. The sensitivity and specificity of average biomarker levels in the likelihood of periodontitis prediction in type 2 diabetes across groups were taken into consideration to provide a more comprehensive evaluation of the performance using a receiver operating characteristic (ROC) curve and further diagnostic test parameters were determined, as shown in Tables 7 and 8. Area under the curve (AUC) was measured to be 0.7567,0.6909 and 0.7555 for SH, SIL6 and SLDH respectively. The cutoff point of ≥3.03 for SH, ≥2.64 for SIL6, ≥7.78 for SLDH showed the optimal balance of sensitivity and specificity (80 % vs. 67 %) for SH, (64 % vs. 62 %), for SIL 6 (65 % vs. 80 %), for SLDH,with positive and negative predictive values of 80 % and 72 %, respectively for SH, of 69 % and 56 %, for SIL6, of 76 % and 63 %, respectively for SLDH.A positive predictive value of 80 % for SH suggested that salivary

Table 1
Comparison of groups for demographics of gender and age and clinical Periodontitis.

	Group 1	%	Group 2	%	Group 3	%	Group 4	%	Total	%	X^2	p-value
Male	23	38.3	37	61.6	21	35	23	38.3	104	43.3	11.13	0.01*
Female	37	61.6	23	38.3	39	65	37	61.6	136	56.6		

Groups	Min	Max	Mean	SD
Healthy	30	57	41.33	7.30
Periodontitis	34	65	49.66	8.84
T2DM with Periodontitis	29	69	48.70	10.64
T2DM with Periodontitis & tooth loss	35	70	55.76	8.03
Total				
F-value	27.068			
P-value	0.0001[a]			

Pair wise comparisons by Tukeys multiple posthoc procedures	
Healthy vs Periodontitis	$p = 0.0001$[a]
Healthy vs T2DM with Periodontitis	$p = 0.0001$[a]
Healthy vs T2DM with Periodontitis & tooth loss	$p = 0.0001$[a]
Periodontitis vs T2DM with Periodontitis	$p = 0.976$
Periodontitis vs T2DM with Periodontitis & tooth loss	$p = 0.001$[a]
T2DM with Periodontitis vs T2DM with Periodontitis & tooth loss	$p = 0.0001$[a]

[a] $p < 0.05$.

Table 2
Comparison of groups for Oral parameters.

	Group 1: Controls	Group 2: CP	Group 3: T2DM with CP	Group 4: T2DM with CP &Tooth Loss	Total	X^2	p-value
Receiving dental check-ups							
Yes	47 (78.3 %)	60 (100 %)	50 (83.3 %)	43 (71.6 %)	200 (83.3 %)	18.960	0.0001[a]
No	13 (21.6 %)	0 (0 %)	10 (17.7 %)	17 (28.3 %)	40 (17.7 %)		
Last Oral Prophylaxis							
≤6 months	8 (13.3 %)	8 (13.3 %)	3 (5 %)	3 (5 %)	22 (9.2 %)	40.187	0.0001[a]
>6 months	29 (48.3 %)	44 (73.3 %)	29 (48.3 %)	16 (26.6 %)	118 (49.2 %)		
Never	23 (38.3 %)	8 (13.3 %)	28 (46.6 %)	41 (68.3 %)	100 (41.6 %)		
Use of oral hygiene aids							
Yes	4 (6.6 %)	6 (10 %)	0 (0 %)	0 (0 %)	10 (4.2 %)	11.270	0.010[a]
No	56 (93.3 %)	54 (90 %)	60 (100 %)	60 (100 %)	230 (95.8 %)		
No of times teeth cleaned per day							
One time	39 (65 %)	43 (71.6 %)	53 (88.3 %)	58 (96.6 %)	193 (80.4 %)	1.7310	0.6300
Two times	21 (35 %)	17 (28.3 %)	7 (11.7 %)	2 (3.4 %)	47 (19.6 %)		
OHI-S							
Good	10 (16.6 %)	4 (6.6 %)	5 (8.3 %)	2 (3.3 %)	21 (8.8 %)	19.46	0.003[a]
Fair	33 (55 %)	26 (43.3 %)	29 (48.3 %)	19 (31.6 %)	107 (44.5 %)		
Poor	17 (28.3 %)	30 (50 %)	26 (43.3 %)	39 (65 %)	112 (46.6 %)		
Gingival Index							
Healthy/Mild gingivitis	53 (88.3 %)	16 (26.7 %)	24 (40 %)	8 (13.3 %)	101 (42.1 %)	85.869	0.0001[a]
Moderate gingivitis	7 (11.7 %)	40 (66.6 %)	35 (58.3 %)	43 (71.7 %)	125 (52.1 %)		
Severe gingivitis	0 (0 %)	4 (6.7 %)	1 (1.7 %)	9 (15 %)	14 (5.8 %)		

[a] $p < 0.05$.

Table 3
Comparison of Average Biomarker Activity by one-way ANOVA across 4 groups with Effect size and Confidence Intervals.

	Average Salivary IL-6(pg/mL) (n = 240)				Average Salivary LDH (IU/L) (n = 240)				Average Salivary Hemin(pg/mL) (n = 240)			
	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD
Healthy((n = 60)	0.04	7.76	2.12	1.46	1.30	9.20	3.28	1.72	0.02	14.22	4.20	2.82
Group 2 (n = 60	0.24	9.61	3.35	2.04	1.26	29.54	5.67	5.43	0.76	41.24	12.98	11.32
Group 3 (n = 60	1.05	11.28	2.93	1.93	1.32	26.15	5.66	4.43	0.83	24.02	10.36	5.86
Group 4 n = 60)	0.10	13.72	4.69	3.35	1.71	44.19	9.21	9.81	2.28	61.11	18.78	14.33
F-value	12.963				9.652				23.453			
P-value	<0.0001*				<0.05*				<0.0001*			

Pairwise comparisons by Tukeys multiple posthoc procedures			
Healthy vs Group 2	P = 0.004*	P = 0.0001*	P = 0.032*
Healthy s vs Group 3	P = 0.049*	P = 0.001*	P = 0.033*
Healthy vs Group 4	P = 0.0001*	P = 0.0001*	P = 0.0001*
Group 2 vs Group 3	P = 0.321*	P = 0.140	P = 0.99
Group 2 vs Group 4	P = 0.002*	P = 0.001*	P = 0.002*
Group 3 vs Group 4	P = 0.0001	P = 0.001*	P = 0.002*

	Sll6	SLDH	SH
Effect Size	0.75	0.92	0.94
95 % Confidence Interval for Mean	2.96–3.59	10.19–12.97	5.144–6.77

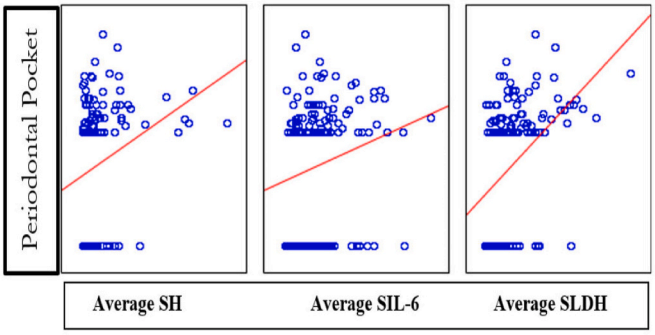

Fig. 1. Scatter plot correlation among average periodontal pocket depth and biomarkers levels.

Hb corresponded to the clinical findings by 80 %, while SIL6 was consistent with 69 % and salivary LDH was consistent with 76 % of the clinical results. The high sensitivity, specificity, and positive and negative predictive values of SH and SLDH suggested the high diagnostic value of the tested method. The AUC also revealed the high diagnostic value of salivary SH, as compared to SIL6 and SLDH for chronic periodontitis in T2DM and tooth loss due to periodontitis. Overall, the positive predictive value was higher for salivary Hb than SIL6 and SLDH, suggesting the acceptable higher discrimination, i.e. ability to identify individuals with and without chronic periodontitis in T2DM was highest

Table 4
Karl Pearson's correlation coefficient depicting direction and degree of relationship of average periodontal pocket depth with biomarker levels.

	Periodontal Pocket Depth		
	r-value	t-value	p-value
Average Hemin	0.4224[b]	7.1901	0.0001[a]
Average IL6	0.1798	2.8202	0.0052[a]
Average LDH	0.2306	3.6558	0.0003[a]

[a] $p < 0.05$.
[b] Pearson correlation coefficient 'r' value showing strong positive correlation.

Table 5
Distribution of percentage of subjects with periodontitis across groups.

	No periodontitis	Mild periodontitis	Moderate periodontitis	Severe periodontitis
Group 1 (n = 60)	60 (100 %)	0 (0 %)	0 (0 %)	0 (0 %)
Group 2 (n = 60)	0 (0 %)	17 (28.3 %)	17 (28.3 %)	26 (43.3 %)
Group 3 (n = 60)	0 (0 %)	22 (36.6 %)	23 (38.3 %)	15 (25 %)
Group 4 (n = 60)	0 (0 %)	5 (8.3 %)	15 (25 %)	40 (66.7 %)

for SH as compared to other biomarkers of this study as depicted in Fig. 2.

4. Discussion

Given its relative ease of collection, flexibility in sampling, ease of transportation, and potential for biobanking, saliva is widely acknowledged as a biological sample with valuable advantages.[17] The focus of research has been on utilizing salivary testing as a non-invasive, cost--effective, and precise diagnostic technique for periodontitis and to bring to light the shortcomings of conventional methods.

According to recent research, inflammatory molecular indicators of periodontal disease can be used to identify "high-risk" individuals who have a higher chance of manifesting the ailment. Undoubtedly, diabetes mellitus is considered one amongst the main risk factors for periodontitis.[18] Potential shared immunoregulatory links is well established between type II DM & periodontal disease further contributing to significant morbidity in the form of tooth loss is seen, which lowers overall quality of life.[19] Consequently, the purpose of this research was to evaluate the significance of salivary hemoglobin, lactate dehydrogenase levels, and interleukin-6 in identifying tooth loss & chronic periodontitis in people with type 2 Diabetes. From a biological perspective, free hemin, which comes from the breakdown of proteins that include heme such as including myoglobin and hemoglobin, is different from that of heme since it involves Fe3+ as opposed to Fe2+. At concentrations of less than 1 μM, free hemin is present in cells because of vascular injury or red blood cell breakdown. It is found in a variety of bodily fluids, including saliva, nasal secretions, urine, CSF, and nasal secretions under pathological conditions (Nam et al., 2015, Nomura et al., 2018, Reed et al., 2015, Segawa et al., 2019, Shimazaki et al., 2011).

Hemin also promotes the growth of bacteria linked to gingivitis (Smalley & Olczak 2017). Free hemin reported as salivary Hemoglobin (SH) in this study is present in the local periodontal environment and is

Table 8
Intercomparison of indicators of chronic periodontitis in type 2 diabetes: Sensitivity and specificity of average biomarker levels.

	Average LDH IU/L	Average IL-6 pg/mL	Average Hemin pg/mL
Cut-off point	≥7.78	≥2.64	≥3.03
AUC	0.7555	0.6909	0.7567
Lower AUC	0.6875	0.6178	0.6875
Upper AUC	0.8103	0.7522	0.8122
Specificity	0.6593	0.6444	0.8000
Sensitivity	0.8000	0.6286	0.6762
Positive predictive value	0.7606	0.6905	0.8091
Negative predictive value	0.6308	0.5614	0.7245
Accuracy	0.7208	0.6375	0.7458

Table 6
Severity of Periodontitis vs Average Salivary Hb, IL-6 & LDH using one way ANOVA.

Severity of periodontitis	Average Salivary IL6 (pg/mL) (n = 240)				Average Salivary LDH (IU/L) (n = 240)				Average Salivary Hemin (pg/mL) (n = 240)			
	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD
No Periodontitis	1.30	9.20	3.21	1.71	0.04	7.76	2.13	1.49	0.02	14.22	4.34	2.78
Mild Periodontitis	1.31	29.54	4.06	4.74	0.24	11.11	2.89	2.35	1.92	24.79	9.33	6.15
Moderate Periodontitis	1.41	18.37	6.08	4.38	0.10	11.28	3.62	2.33	0.76	36.15	11.78	8.35
Severe Periodontitis	1.26	44.19	8.87	8.86	0.19	13.72	4.07	2.85	0.38	61.11	17.82	14.19
F-value	11.9177				8.4260				22.9334			
P-value	0.0001*				0.0001*				0.0001*			
Pair wise comparisons by Tukeys multiple posthoc procedures												
No Periodontitis with Mild	p = 0.8951				p = 0.3612				p = 0.0471*			
No Periodontitis with Moderate	p = 0.0560				p = 0.0044*				p = 0.0003*			
No Periodontitis with Severe	p = 0.0001*				p = 0.0001*				p = 0.0001*			
Mild Periodontitis with Moderate	p = 0.3405				p = 0.4199				p = 0.5908			
Mild with Severe Periodontitis	p = 0.0001*				p = 0.0367*				p = 0.0001*			
Moderate with Severe Periodontitis	p = 0.0387*				p = 0.6946				p = 0.0020*			

Table 7
Indicators of chronic periodontitis in type 2 diabetes: Sensitivity and specificity of average biomarker levels across groups.

	Cut-off point	AUC	Specificity	Sensitivity	Positive Predictive Value	Negative Predictive value	Accuracy
Group 2							
SLDH	≥7.63	0.7300	0.6000	0.7000	0.667	0.6310	0.6530
SIL6	≥2.31	0.7672	0.7143	0.5000	0.7692	0.4286	0.6500
SH	≥1.59	0.6958	0.9048	0.6671	0.7170	0.4286	0.6833
Group 3							
SLDH	≥11.49	0.8333	0.8750	0.8333	0.7778	0.1667	0.8500
SIL6	≥2.85	0.4510	0.4211	0.5000	0.5926	0.3333	0.4500
SH	≥2.37	0.7333	0.9211	0.4545	0.7447	0.7692	0.7500
Group 4							
SLDH	≥15.46	0.6667	0.7059	0.6667	0.9231	0.0000	0.7000
SIL6	≥4.00	0.4400	0.4364	0.4000	0.8889	0.0606	0.4333
SH	≥3.60	0.7855	0.8000	0.6000	0.9565	0.2143	0.7833

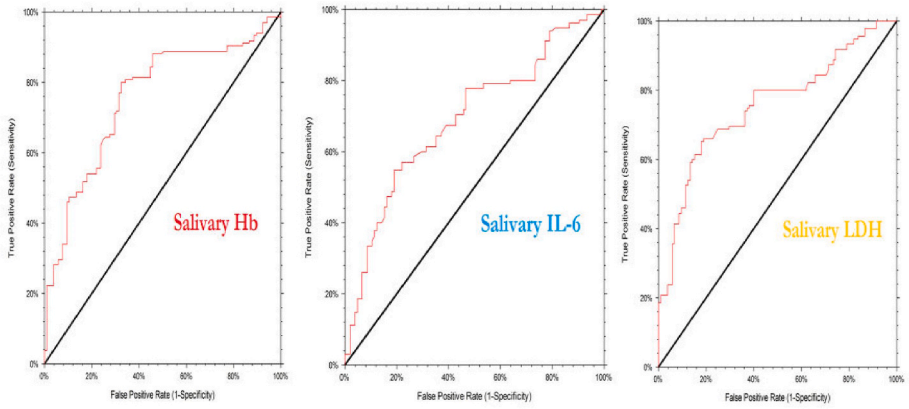

Fig. 2. ROC Curve representing the sensitivity and specificity of Average Biomarker levels in the prediction of Chronic Periodontitis.

possibly a significant component contributing to the progression of periodontal disease. When free hemoglobin is present in large quantities, it catalyzes the non-enzymatic production of reactive oxygen species, which results in oxidative damage. To intensify the inflammatory process, it seems to control vascular permeability, endothelial cell adhesion, leucocyte recruitment, and apoptosis.Iron and hemin control a number of potential virulence factors for bacteria. Through increased macrophage production of proinflammatory cytokines, such as interleukin (IL)-1b, IL-6, IL-8, and tumour necrosis factora, hemoglobin in the gingival crevicular fluid potentiates the pathogenic effects of P. gingivalis and lipopolysaccharide from periodontopathogens.As a result, heme is responsible of boosting virulence factors to cause host destruction.[20] The primary sources of heme for P. gingivalis in vivo include erythrocytes, gingival crevicular fluid, and hemoproteins found in saliva. P. gingivalis uses several ways to obtain heme. The ones that use gingipains, hemolysins, and hemagglutinins are the best defined among them.[21]

A periodontal pocket is referred to as a pathologically deepened gingival sulcus. The extent of periodontal disease status in the entire oral cavity can be described as the number of sites having a pocket probing depth of 4 mm or more.The gingival index is the quantitative and qualitative measure of the status of gingival inflammation scored from 0 to 3 which can be used in statistical analysis. As investigated in previous studies, the current study did find the gingival index across four groups to be statistically significant underlining the clinical association of severity of gingivitis with periodontitis.[22]The gradual variation of the severity of gingivitis ranging from mild to severe across the four groups considered in this study can be associated with the HbA1c levels across the groups. Therefore the screening for gingivitis can be used as a tool to alleviate the established risk of periodontitis in type II diabetics. It should be noted that the OHI-S score across the four groups was statistically significant despite the observed significant difference in gingival index across the groups suggesting the presence of systemic inflammation from the disease condition outweighs the effect of local factors (debris and calculus). In this study we found that although 83.3 % had previous dental checkups, proportion receiving oral prophylaxis was 9.2% (last 6 months), 49.2 % (more than 6 months ago), and 41.6 % (never) indicating subpar levels of compliance and awareness towards oral hygiene practices. Therefore, the finding that glycemic control as a strategy for periodontal wellness can be confirmed with a larger sample size. Multicentre studies are suggested in the future to address the generalizability with regard to specific demographic characteristics.

In this study to precisely determine the maximal destruction for each tooth, CAL and probing depth were assessed at six different spots per tooth in this investigation. The APPD was developed as a metric to measure the depth of the periodontal pocket in the oral cavity, which is then utilized in statistical analysis. Due of their greater accuracy, the CDC-AAP case definitions were considered than AAP/European Federation of Periodontology (EFP) case definitions as evidenced by two cross-sectional studies involving adolescents and adults by Morales et al.[23] Noticeably in this study Biomarkers levels were found markedly higher in severe periodontitis indicating their discriminating ability of disease progression.

Statistically significant variation was observed when the average values of each biomarker activity were compared across the four groups using a one-way ANOVA, with Average Salivary IL-6, Average Salivary LDH, Average Salivary Hemin activity ($F = 12.963$) ($p < 0.0001$), ($F = 9.652$) ($p < 0.05$). ($F = 23.453$) ($p < 0.0001$) respectively indicating that there were differences in the mean average biomarker levels among the four groups for each biomarker. To identify the pairwise comparison between the four groups, Tukey's multiple post hoc analysis was used, and the results showed that there was a significant difference between group I (controls) vs group IV, group II vs group IV, and group III vs group IV.

It was implied that group I (controls) had the lowest mean average biomarker levels and group IV had the highest levels since there was no appreciable difference between groups I and II and groups I and III. The raised biomarker levels within the Group 4 indicates a direct association with increased severity of clinical periodontitis.

This study assessed the oral cavity's site-specific release of biomarker levels. Clinical examinations were conducted by a calibrated examiner and laboratory assessments were conducted in triplicate with appropriate controls to ensure replication and robust statistical analyses. Average levels, which are calculated by dividing the total number of teeth by the sum of the levels of each biomarker in saliva, were examined for significance and applicability in this investigation. The results revealed that average biomarker levels had greater relevance in the detection of CP than the total values of salivary biomarkers.[14]

To eliminate fluctuations brought on by circadian rhythm, saliva collection among subjects was standardised between 8am and 10am[24] and unstimulated saliva sample collected during fasting. More than two thirds of diabetics have hypertension.[25] There exists a lack of consensus regarding the causal relationship between hypertension and periodontitis[26] with studies indicating a significant proportion of asymptomatic stage I hypertensives.[27] In our study, only 15 % of the subjects with diabetes (i.e., group III and group IV) had mild HTN (140–159/90–99 mm Hg) as a co-occurring condition with type 2 diabetes. Considering these data, we took into consideration the extremely low probability that HTN causes periodontitis in our investigation.

The specificity and sensitivity of SH[11] SLDH[28] and SIL-6[29] have been assessed in research to ascertain the clinical applicability. Comparison of these diagnostic test parameters using colorimetric tests in this research suggested acceptable reasonable discrimination, i.e. ability to diagnose subjects with and without chronic periodontitis in T2DM. Considering the three analytes across groups, SLDH demonstrated a specificity of 0.6, 0.8 and 0.7 while SIL-6 of 0.7, 0.4, 0.4 and SH with 0.9, 0.9 and 0.8

across Group 2, Group 3 and Group 4 respectively as depicted in Table 7. For a more comprehensive assessment of the performance, a plot was made of the receiver operating characteristic (ROC) curve of the Three biomarkers were considered in this research to predict the likelihood of periodontal disease in people with Type 2 Diabetes. SH ranged higher in its specificity to SLDH and SIL-6 while SLDH ranked higher in its sensitivity as depicted in Table 5 and Fig. 2.

Comparing the results of diagnostic ability with other studies, study by Nomura et al., 2006 concluded that LDH level had the highest sensitivity and specificity of 0.66; specificity 0.67 for screening of periodontitis with salivary enzyme tests. Another study for a screening method as an alternative to the community periodontal index for Periodontitis by the same author in 2016 inferred the sensitivity and specificity for Salivary hemoglobin and lactate dehydrogenase levels were 0.722 and 0.711 respectively and the authors stated that by combining both the biomarkers positive predictive value was 91.7 % assuring higher diagnostic validity. Multivariate logistic regression analysis in a study for screening poor periodontal status by Shimazaki et al. (2011) using salivary occult blood test demonstrated a sensitivity and specificity of 0.72 and 0.52, respectively.

These findings are in line with results of the present study supporting the diagnostic value of SLDH and SH in chronic Periodontitis. Study by Ebersole et al. (2015) to elicit performance measures of Salivary concentrations of IL-1β,IL-6,MMP-8, in combination by pairing the biomarkers including IL-1β/IL-6,IL-1β/MMP-8,and IL-6/MMP demonstrated the sensitivity and specificity values approximating 0.8. In this study although SIl6 showed an overall specificity and sensitivity of 0.64 and 0.62 respectively, AUC ranged less than 0.5 and diagnostic ability in Group 3 and Group 4 was found inadequate as depicted in Table 7. Contradicting the results of this and previous studies in literature, study by Moghadam S et al. (2022) for the biomarkers Salivary LDH and HbA1C did not show adequate sensitivity or specificity for screening chronic Periodontitis and hence stated that LDH and HbA1c, cannot be used with certainty for screening chronic periodontitis. Incidentally our study has pioneered to consider the biomarkers in conditions of T2DM, hence there are no available studies in literature till date for comparison of the diagnostic value of biomarkers for chronic periodontitis in this systemic condition.

This study according to authors is a Pioneer Research in evaluating role of salivary hemoglobin as a biomarker with Indian T2DM population and is funded by ICMR New Delhi. Study results enhance the validity of non-invasive periodontitis detection method. Research investigates predictive performance of Biomarkers amongst global health burden - Periodontal illness and T2DM. Saliva and its analytes are widely used as a crucial diagnostic specimen for hormones, medications, antibodies, and forensic cases. The design of longterm studies evaluating interventional techniques will be further advanced using collected saliva in automated biochemical test formats. Standardizing methods or validating results with multiple kits to address this limitation is advocated.

4.1. Limitations and Scope

Among the study's practical constraints was age and gender matching and that it was performed at one time point in a specific clinical context. A prospective investigation with a larger sample-size, multicentred with several assessments made at multiple time points can better assess the periodontium's response to hyperglycemia. Additional investigation into chair-side, point-of-care (POC) Diagnostics with microfluidic & Lab-on-a-chip technology could greatly adjunct and the advance routine, remote screening for CP in T2DM. Comparison of study parameters with other biomarkers and other Salivary Hb measuring methods can justify the predictive power of the salivary hemoglobin levels towards its diagnostic accuracy. Outcome measures of with Cut off values stratified by gender, compliance with dental hygiene practices, age and the number of teeth remaining, post-periodontal therapy, T2DM duration and Rx and Fixed prosthesis vs. Removable prosthesis, Role of oral microbiome in CP with & without T2DM and analysis of other inflammatory mediators in T2DM and CP can be researched. Longitudinal studies are suggested in future which could possibly establish a temporal relationship between biomarkers and periodontal disease progression. Role of oral microbiome in CP with & without T2DM and analysis of other inflammatory mediators in T2DM and CP can be researched.

5. Conclusion

The ability to discriminate between the degree of development of periodontitis with tooth loss and elevated glycemic levels can be greatly aided by salivary hemoglobin. Salivary Hemoglobin can prove a viable biomarker by incorporating standardised saliva collection method when incorporated in medical check-up system for identifying conditions requiring collaborative care and establish referral networks. With advancing technology and further research into the limitations of this study, Salivary Hemoglobin seems a promising biomarker that can be recommended as a dental chair side test involving oral health care with systemic health promotion. As a low-cost method alternative for oral examinations, we can forsee it as a visual semi-quantitative assessment using lateral flow analysis of heme from saliva samples that can be used for patient education at remote settings with limited access for laboratory facility supporting at-home sampling. In general practice and dental clinics, routine use guarantees non-invasive early detection and comprehensive interdisciplinary care.

Conflict of interest

We declare no conflict of Interest among authors and accept all the terms and conditions of journal.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jobcr.2024.08.002.

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