Tracing Salivary NET-Derived CitH3 as a Diagnostic Biomarker in Periodontal Disease: A Case Control Study

Mohammedalmustafa Qasim Abdul-Hussien, and Ghasaq Asim Abdul-Wahab

Department of Oral Surgery and Periodontology, College of Dentistry, Al-Mustansiriyah University, Baghdad, Iraq.

Abstract

Periodontal diseases encompass a continuum of inflammatory conditions, as a result of altered host-microbe interactions. Among the immune cells involved, neutrophils are pivotal through their ability to generate web-like structures released by neutrophils called (NETs) and histone H3 proteins modified through citrullination (CitH3). Thus, the study aim was based on the evaluation of diagnostic potential of salivary CitH3 in patients with periodontal disease. Ninety participants aged from 25 to 55 years involved in the study. Divided equally into three groups, healthy controls, patients with gingivitis, and patients with severe periodontitis/stage III. The periodontal parameters were recorded and salivary samples collected for (CitH3) evaluation using ELISA. Kruskal-Wallis, Spearman's correlation, and ROC curves were used for statistical analysis. CitH3 showed significantly higher levels in gingivitis and periodontitis groups ($P < 0.001$), with a positive significant relationship regarding gingival index. The receiver operating characteristic (ROC) analysis demonstrated that CitH3 exhibited a high level of discriminative power between gingivitis and control subjects (AUC = 0.90) and a decent diagnostic ability in differentiating periodontitis from health (AUC = 0.86). Salivary CitH3 seems to be a useful biomarker for periodontal inflammation because it shows how NETosis plays a role in the disease getting worse. The observed increase in patient groups substantiates its diagnostic significance, while its association with gingival inflammation suggests a potential function as a prognostic marker for periodontal disease activity.

Keywords: Periodontitis, Gingivitis, Saliva, NETosis.

1. Introduction

Periodontitis is a chronic inflammation of tissues that support teeth. Although it begins with microbial biofilm accumulation, it advances mostly due to the host's immune response, activating gingival inflammation, pocket deepening, loss of attachment, and alveolar bone destruction. Diseases process involves a complex interaction between host defences and microbial variables, with neutrophils playing an essential role in shaping the outcome [1].

Neutrophils combat pathogens by either killing them intracellularly or releasing antimicrobials through massive web-like structures called neutrophil extracellular traps (NETs) through a cell death process known as NETosis. NETs are made up of decondensed chromatin and antimicrobial substances and are mostly used against substantial pathogens like fungus, parasites, and bacterial microbes that cannot be removed intracellularly.

Dysregulated NETosis can be deleterious because NETs are a significant reservoir of extracellular histones. The citrullination of histone H3 (CitH3), promoted by peptidyl arginine deiminases, is a distinctive feature of NETosis. This procedure produces CitH3. This molecule has been found in saliva and is increasingly regarded as a possible indicator of both neutrophil activity and periodontal inflammation [2, 3].

Histones are toxic to bacteria and host cells, but they can also cause inflammation at low doses by activating macrophages via toll-like receptor 4 (TLR4) and inducing cytokines [2]. Recent studies have demonstrated that an increase in CitH3 levels suggests the presence of an inflammatory condition such as septicaemia, autoimmune disorders, correlates positively with extent of disease and destruction of tissue [4, 5].

Salivary CitH3 serves as a non-invasive biomarker for neutrophil activity in periodontal disease, perhaps assisting in the assessment of disease progression from gingivitis to advanced periodontitis. The objective of the present study is to investigate the essential function of neutrophils and NETs in periodontal inflammation by evaluating salivary CitH3 levels and test the potential diagnostic and prognostic ability.

2. Materials and Methods

This was an observational case control study conducted in Baghdad between December 2024 and May 2025. Participants included in this study were individuals seeking care from the periodontal department. All of them given a detailed information regarding the study's goals. The study conforms with Helsinki declaration.

Participants will be required to complete a consent form that has been approved by the College of Dentistry's ethics committee under the reference number REC186 and study number

MUOSU-202126. The sample size was determined by Using G power (authored by Franz-Faull, University of Kiel, Germany) with a power of 0.90 and a two-sided alpha error of probability of 0.05, an effect size of F is 0.4 (large effect size) [6, 7]. Under this condition the required sample size is 84, the sample size was expanded to 90.

This sample consisted of 90 individuals aged from 25 to 55 years, divided into three groups as follows. Group I represented the control group, which included 30 volunteers with clinically healthy periodontium, no (CAL), (PPD) ≤ 3 mm, and (BOP) less than 10% [8]. Group II consisted of 30 patients with gingivitis (no CAL), PPD ≤ 3 mm, (BOP) > 10% [8]. Group III included patients with severe periodontitis (stage III).

The study inclusion criteria included patients with detectable interdental (CAL) on ≥ 2 non-adjacent teeth, or the presence of ≥ 5mm CAL on the buccal (facial) or lingual/palatal surfaces associated with pocketing ≥ 6 mm at ≥ two teeth [9]. In addition, all periodontitis cases were generalized and unstable, with no risk factors, e.g., Diabetes mellitus (DM) and/or smoking.

All participants were required to demonstrate excellent general health, be free of any preexisting systemic conditions, and have a minimum of 20 teeth. Patients were excluded from the sample if they had an oral inflammatory disease (other than periodontitis) that might affect the levels of the biomarker under study. Individuals with systemic diseases, a history of alcohol or smoking, pregnant or breastfeeding women, individuals who had taken anti-inflammatory medications within the previous three months, or those who had undergone extensive periodontal treatment and were using vitamins, antioxidants, or contraceptives were also excluded from this study.

The indices used to assess the periodontal health of participants were: PLI, GI, % of BOP, PPD, and CAL, and measurements were recorded using a Williams periodontal probe. Tooth surfaces were examined on six different surfaces, mesiolingual, midlingual, distal lingual, distal buccal., mid-buccal, and mesio-buccal. The third molars were excluded.

2.1 Saliva Sampling

Unstimulated saliva sample of about 5 mL was collected from patients and controls using the passive drooling method between 9:00 a.m. and 12:00 p.m. Participants were advised not to eat or drink except water for at least an hour before the saliva sampling. For standardize conditions and lower the risk of contamination, a whole, sampling was performed prior to any oral clinical examination.

Each participant was asked to allow their saliva to accumulate at the bottom of their mouth and then passively drool into a sterile plastic container without swallowing during the procedure [10]. After collection, samples were centrifuged at 3,000 rpm for 10 minutes, and the clear upper fluid was carefully separated.

Maintaining the integrity of biomarkers and prevent bacterial growth, collected samples were stored at -20 °C from collection until the ELISA test. Prior to analysis, frozen samples were thawed to room temperature.

2.2 ELISA Detection of Salivary (CitH3)

The ELISA technique for estimation of Cit.H3 was a sandwich technique (My BioSource/USA; Catalog No.; MBS1609017).

2.3 Statistical Work

Data analysis done using SPSS software, version 28, released in the United States. The minimum ("min") and maximum ("max") of the data were calculated, along with the arithmetic mean, standard deviation, and standard error. Normality was tested with Shapiro-Wilk, showing non-normal distribution.

Group comparisons used Kruskal-Wallis, followed by Dunnett's tests for intergroup comparison. The ROC (robust operating characteristic) and AUC (area under the curve) tests were used. Statistically significant results considered when P -value ≤ 0.05.

3. Results

Regarding the demographic characteristics and characteristics of the sample participating in the survey (table 1) indicates the absence of a statistically significant indicator for age (P = 0.3). Similarly, although males were more common in all groups, differences in gender distribution did not reach statistically significant values (P = 0.3).

Table 1: Age and gender of the study population.

All groups		Group I	Group II	Group III	P-value
Age					
Mean ± SD	46.1 ± 3.5	45.2 ± 2.3	46.2 ± 2.4	47.1 ± 4.9	0.3 NS
SE	0.3	0.4	0.4	0.8	
Gender					
Male	54 (60 %)	16 (53.3 %)	17 (56.7 %)	21 (70 %)	0.3 NS
Female	36 (40 %)	14 (46.7 %)	13 (43.3 %)	9 (30%)	

SD: Standard Deviation, SE: Standard Error, NS: Non-Significant, * Kruskal-Wallis Test

3.1 Clinical Periodontal Parameters

Highly Significant differences were found in the teeth loss percentage, PLI, GI, and BOP %, (P < 0.001) among study groups. Measurements of (PPD) and (CAL) were restricted to Group III, since only this group comprised patients with severe periodontitis (table 2).

Table 2: Clinical Periodontal Parameters.

Parameters	Group I N. 30		Group II N. 30		Group III N. 30		P value
	Mean ± SD	Median	Mean ± SD	Median	Mean ± SD	Median	
No. lost teeth (%)	12 (1.5 %)		76 (9.1 %)		143 (17.1 %)		< 0.001 HS
PLI	0.2 ± 0.09	0.2	1.5 ± 0.7	1.5	2.5 ± 0.2	2.5	< 0.001 HS
GI	0.2 ± 0.06	0.3	2.3 ± 0.4	2.2	2.6 ± 0.1	2.6	< 0.001 HS
BOP (% of score 1)	4.3 %		51.4 %		69.0 %		< 0.001 HS
PPD	-	-	-	-	9.0 ± 0.7	6.9	-
CAL	-	-	-	-	6.0 ± 0.7	5.8	-

PLI: Plaque Index; GI: Gingival Index; BOP: Bleeding on Probing; PPD: Probing Pocket Depth; CAL: Clinical Attachment Loss; N., Number; SD: Standard Deviation; HS: Highly Significant, * Kruskal-Wallis Test, **Chi Square test for percentage.

3.2 Salivary Level of (CitH3) and The Correlation with Clinical Parameters

The current study results revealed that salivary CitH3 were highly significant (P < 0.001). Patients with gingivitis and periodontitis demonstrated markedly elevated CitH3 values of 102.3 ± 19.3 µg/mL and 101.0 ± 23.3 µg/mL respectively compared to healthy controls, as illustrated in (table 3).

Table 3: Descriptive and Inferential Statistics of Salivary CitH3.

Parameter	Group	No.	Mean ± SD	Median	Min.-Max.	SE	P value
CitH3 µg/mL	I	30	66.2 ± 21.8	64.9	10.6-99.5	21.8	<0.001 HS
	II	30	102.3 ±19.3	102.5	52.9-147.3	19.3	
	III	30	101 ± 23.3	99.7	71.2-160.7	23.3	

CitH3: Citrullinated Histone 3, No: Number, SD: Standard Deviation, SE: Standard Error Min: Minimum; Max: Maximum; HS: Highly Significant، *Kruskal-Wallis Test.

Pairwise comparisons revealed that controls had significantly lowest CitH3 levels than patient groups (P < 0.001), with a non-significant difference between gingivitis and severe periodontitis groups (P = 1.0), (table 4).

Table 4: Pairwise comparison of CitH3 among the study groups

Parameter	Group	MD	SE	P value
Cit.H3 µg/ml	I- II	-32.6	6.7	<0.001 HS
	I- III	-36.9	6.7	<0.001 HS
	II- III	4.3	6.7	1.0 NS

CitH3: Citrullinated Histone 3, MD: Mean Difference, SE: Standard Error, NS: Significant; HS: Highly Significant, *Dunnett post hoc test.

3.3 The Correlation Between Salivary CitH3 Levels and Clinical Periodontal Parameters

Regarding the correlation of CitH3 level and periodontal parameters, there was a significant positive association (r = 0.3) at a p value of (0.05) encountered with GI in patients with severe periodontitis. While there were non-significant correlations regarding the rest of parameters (P > 0.05) as listed in (table 5).

Table 5: The Correlation between CitH3 levels and Clinical Periodontal Parameters.

Parameters	Cit.H3					
	Group I		Group II		Group III	
	R	P value	R	P value	r	P value
No. of lost teeth	-0.2	0.1	0.1	0.6	-0.1	0.3
PLI	-0.1	0.6	-0.01	0.9	0.2	0.2
GI	-0.1	0.6	0.1	0.5	0.3	0.05 S
BOP 1	-0.3	0.1	-0.2	0.2	-0.02	0.8
PPD	-	-	-	-	-0.06	0.7
CAL	-	-	-	-	0.01	0.9

r: Spearman correlation, PLI: Plaque Index, GI: Gingival Index, BOP: Bleeding on Probing, PPD: Probing Pocket Depth, CAL: Clinical Attachment Loss; NS: Non-Significant, S: Significant

The diagnostic accuracy of salivary CitH3 in differentiating controls from patients with gingivitis and periodontitis, showed a high diagnostic performance when comparing healthy people to the gingivitis group, with an AUC of 0.90 (P < 0.001). At a cutoff value of 84.9, it had 90 % sensitivity and 84% specificity. In the comparison between healthy controls and severe periodontitis patients, CitH3 showed good accuracy with an AUC of 0.86 (P < 0.001), and at a cutoff point of 81.0 sensitivity and specificity were 76 % and 67 % respectively. From results before, it can be noted that CitH3 is able to distinguish and assess the disease condition, whether it is in its early stages or the situation is deterioration (table 6).

Table 6: Diagnostic performance of CitH3 in differentiating oral health, gingivitis, and severe periodontitis based on ROC curve analysis.

Biomarkers	GR	AUC	P-value	Optimal cutoff point	Sensitivity	Specificity
Cit.H3	GR I–GRII	0.9	<0.001 HS	84.9	0.9	0.84
Cit.H3	GR I–GRIII	0.86	<0.001 HS	81.0	0.76	0.67

4. Discussion

Based on previous studies, NETs are a prerequisite and a key factor in the development of periodontal disease. Their uncontrolled, random activity causes tissue deterioration and inflammation [2, 3]. The current study observed a significant increase in the concentrations of CitH3 in patients with periodontal diseases. This is consistent with previous studies, that showed that CitH3 associated with chronic immune diseases [4, 5].

Concerning the demographic features of the study sample, a non-significant difference in both age and gender was noted suggesting matched study groups. Patients with periodontal diseases consistently shown substantial increases in PLI, GI, and BOP %, highlighting the essential involvement of dental plaque biofilms as the principal mediators of gingival inflammation and periodontal degradation [11-13].

These results correspond with recent data demonstrating that inadequate plaque control is significantly associated with increased gingival inflammation and the progressive deterioration of periodontal tissue [14, 12]. The present study demonstrated a significant increase in salivary (CitH3) levels across the study groups. The lowest concentration recorded by controls, while the groups with gingivitis and severe periodontitis showed much higher levels, with a non-significant difference between gingivitis and periodontitis groups.

CitH3 levels increase rapidly with the onset of gingival inflammation and stay elevated as the condition deteriorates. This trend indicates that CitH3 signifies acute inflammatory activity rather than the extent of structural periodontal damage. In accordance with our findings, Papayannopoulos [2], and Knight et al. [3] emphasized that NETosis-related biomarkers, such as CitH3, are intricately linked to neutrophil-mediated inflammatory responses rather than to tissue damage. Yipp and Kubes; Thieme et al. all demonstrated that increased of CitH3 levels correlate with the severity of inflammatory burden in systemic diseases, such as sepsis and autoimmune disorders [4,15].

Moreover, König et al. found that NETosis markers exhibited a stronger correlation with gingival inflammation markers than with loss of attachment [16]. Conversely, numerous studies have demonstrated a more extensive function of NET-derived markers, indicating their correlations with pocket depth and attachment loss in severe cases [17,18]. The reason for these varying results may be attributed to variations in the stage of the disease, the characteristics of the population, or the methods employed.

In general, the study results suggest that salivary CitH3 is a sensitive indicator of inflammatory activity in periodontal disease, particularly during the early and active phases of gingival inflammation. In patients with severe periodontitis, salivary CitH3 exhibited a strong positive correlation with the GI, while no significant correlations were identified with attachment loss or probing pocket depth.

This indicates that CitH3 signifies persistent gingival inflammation rather than structural damage, aligning with studies that demonstrate biomarkers associated with NETosis exhibit a stronger correlation with acute inflammatory alterations than with chronic periodontal deterioration [16]. ROC curve analysis showed that CitH3 was useful for diagnosing diseases. It was moderately accurate at separating periodontitis from health (AUC = 0.86, sensitivity = 76 %, specificity = 67 %) and very accurate at separating gingivitis from healthy people (AUC = 0.90, sensitivity = 90 %, specificity = 84 %). These findings suggested that CitH3 might serve as an important biomarker for monitoring the progression of illness and evaluating periodontal health.

Overall, the study's findings supported CitH3 is a promising salivary biomarker that demonstrated neutrophil and NETosis activity as the primary drivers of inflammation. Further research is mandatory to explore the specific underlying mechanisms between NET markers and microbiomes in different stages of periodontitis could offer important insights into the link between NETosis and periodontal diseases.

5. Conclusion

Salivary CitH3 levels were markedly elevated in patients with gingivitis and severe periodontitis compared to healthy individuals. Its diagnostic accuracy varied from good to outstanding in differentiating health from disease, demonstrating its potential as a dependable non-invasive biomarker. Results indicated that CitH3 may serve as an effective marker for early detection of periodontal inflammation and disease progression monitoring.

6. Acknowledgment

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7. References

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