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Evaluation of galectin-3 and peptidyl arginine deiminase-4 levels in saliva for periodontal health, gingivitis and periodontitis

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Abstract:

Background: Comparing the saliva levels of galectin-3, peptidylarginine deiminase 4 (PAD4) in individuals with periodontitis and gingivitis and with healthy periodontium was the purpose of this clinical research.

Methods: This study was an observational case-control study which was conducted at Al- Aamiriya Specialized Dental Center and Department of periodontics, College of Dentistry, University of baghdad from August, 2024 to November, 2024.

(84) systemically healthy and non-smoker individuals consisting of periodontitis (group PD/n = 28), gingivitis (group G/n = 28), and periodontally healthy (group H/n = 28) were recruited for this research. Clinical parameters such as probing depth, clinical attachment level, gingival index, plaque index, and bleeding on probing were recorded in periodontal charts. Enzyme-linked immunosorbent assay method was used in evaluating the saliva levels of galectin-3 and PAD4 for study groups.

Results: The saliva galectin-3 total amount was highest in group G3 compared with group G1 and group G2 ($p<0.05$) and in G2 higher than G1.The saliva PAD-4 total amounts were highest in group G3 compared with group G1 and group G2 ($p<0.05$) and in G2 higher than G1.

Conclusions: Galectin-3 and PAD4 may be involved in the periodontal disease pathogenesis considering the elevated levels of these molecules in periodontal disease. These biomarkers may be used in the diagnosis of periodontal diseases.

Keywords: galectin 3, salivary fluid, periodontal diseases, protein-arginine deiminases, peptidylarginine deiminase.

1 . INTRODUCTION:

The classical presentation of periodontal disease is progressive inflammatory events leading to damage of periodontal soft and hard tissues in susceptible patients (Imran et al., 2024). Initiated by dental plaque and modified by environmental and genetic risk factors (Dahash and Mahmood, 2019). Periodontitis (CP) was found to be higher in the presence of some risk factors like diabetes (Ibraheem et al., 2020).

In the periodontology field, researchers are looking for useful diagnostic biomarkers that could point to the development of a disease process in a periodontium before clinical periodontal destruction becomes too extensive. Identifying these useful biomarkers is an important issue because clinical parameters and radiographical findings used in the diagnosis of pathologies involving the periodontium provide only past information for the breakdown of the periodontal supporting tissues, doesn't explain the present situation for the disease activity, and doesn't estimate the future progression of periodontal disease (Buduneli N, Kinane DF, 2011).

Galectins, a group of mammalian lectins, are essential for cellular homeostasis throughout the immunological response. Extracellular or intracellular galectins significantly influence cell differentiation, proliferation, adhesion, survival, apoptosis, and signaling pathways (Barut A., Z., et al., 2023). Galectin-3 (Gal-3) is secreted by various cell types, including eosinophils, macrophages, monocytes, natural killer cells, dendritic cells, and T or B lymphocytes. Moreover, certain research have asserted that Gal-3 directly interacts with pathogen-associated molecular patterns, which are crucial for host defense against microorganisms (G.R. Vasta, 2012).

Saliva contains various significant inorganic and organic compounds that serve as non-invasive diagnostic agents. Due to its ease, cost-effectiveness, painlessness, and safety, saliva collection is favored by both patients and healthcare professionals. Consequently, salivary analysis (Sha et al., 2024; Mohammed et al., 2022) is advised for the diagnosis and monitoring of several oral and systemic conditions, including cancer, infections, and cardiovascular illnesses (S. Chojnowska et al., 2018; M. Boroumand, A et al., 2021). Periodontitis and gingivitis are marginally linked to cardiovascular, cardiometabolic, autoimmune illnesses, and mental health disorders (D.T. Zemedikun et al., 2021). Complex pathological processes in various conditions may modify saliva -omics, which are crucial for the identification of systemic diseases or localized pathologies (G. Baima et al., 2021; E. Kaufman, I.B. Lamster, 2000). This bodily fluid may serve as a diagnostic tool for assessing the development and diagnosis of periodontal disease (E. Kaufman and I.B. Lamster, 2000).Gal-3 was identified in many dental conditions, including periapical granuloma, radicular cysts, and gingival disorders (M. Karsiyaka Hendek et al., 2021). Recent investigations indicated elevated Gal-3 levels in serum or gingival crevicular fluid (GCF) of individuals with gingivitis or periodontitis (M. Karsiyaka Hendek et al., 2021).

Prior research examined the biomarker in gingival crevicular fluid, serum, or the periodontal ligament (M. Karsiyaka Hendek et al., 2021). This study aims to assess the amounts of Gal-3 and PAD4 in saliva, a method that is straightforward and convenient for collection. This study aims to examine the levels of Gal-3 and PAD4 in the saliva of patients with periodontitis, gingivitis, and periodontal health.

The conversion of arginine residues to citrulline residues, termed citrullination (a Ca2+-dependent process), is executed by enzymes known as peptidylarginine deiminases (PADs) (Vossenaar ER, 2003). The citrullination process maintains homeostatic functions and is also involved in autoimmune disorders and inflammatory situations (Neidhart M et al., 2005). Anti-citrullinated protein antibodies (ACPAs) are antibodies generated in response to citrullinated proteins. They occur in 70% of patients with rheumatoid arthritis and are associated with joint deterioration and severe illness (Meyer O,

2003). The citrullinated proteins present in tissues affected by periodontitis and synovial tissue samples from rheumatoid arthritis (RA) exhibit significant similarity, with periodontitis considered a potential inducer of anti-citrullinated protein antibodies (ACPA) production (Nesse W et al., 2012). To date, five members of this enzyme family (PAD1, peptidylarginine deiminase 4 [PAD4], and PAD6) have been found in humans (Vossenaar ER, 2003). PAD4 is predominantly sourced from macrophages and neutrophils, contributing to the development of rheumatoid arthritis (Senolt L and Nielsen CH, 2016). A clinical investigation revealed elevated PAD activity in patients with periodontitis, regardless of the presence of RA (Laugisch O, 2016).

Engström et al. demonstrated that PAD4 expression was elevated in the gingival tissue of periodontitis patients relative to healthy controls. The authors suggested that this circumstance appeared to be unrelated to the presence of periodontopathogens, including Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis (Engstrom M et al., 2018). In an immunohistochemical study, the PAD4 enzyme was detected in 14 out of 15 periodontal tissue biopsies (93%) from patients diagnosed with periodontitis (Janssen KMJ, 2017).

Upon reviewing the literature, no study has comparatively analyzed the levels of galectin-3 and PAD4 in saliva across patients with periodontitis, gingivitis, and a healthy periodontium. The synthesis of the aforementioned investigations (Laugisch O, 2016; Janssen KMJ, 2017) posits the hypothesis that levels of galectin-3 and PAD4 are elevated in persons with periodontal disease compared to those with clinically healthy periodontium. This clinical study aims to investigate salivary levels of galectin-3 and PAD4 in periodontitis, gingivitis, and healthy control groups, while also studying the relationships between biochemical and clinical parameters.

2 .MATERIALS AND METHODS:

This study was an observational case-control study which was conducted at Al- Aamiriya Specialized Dental Center and Department of periodontics, College of Dentistry, University of baghdad from August, 2024 to November, 2024.

This study included 84 participants who were systemically healthy, including 28 healthy periodontium (control group), 28 gingivitis and 28 periodontitis (cases). The specific case sheet necessitates the inclusion of the individual's name, age (≥ 18 years old), year, and gender (either "male" or "female").

Individuals with systemic diseases, chronic disease medications, tobacco use, previous periodontal interventions, and pregnant women were not included.

After the collection and separation of saliva , Saliva samples were analyzed for GAL-3 and PAD-4 levels using ELISA assays , saliva samples transferred to centrifuge tubes using sterile syringes. The samples were vortexed for 10 min at room temperature to eliminate cells and food debris and to diminish the turbidity of the saliva to protect the accuracy of the analysis(E. Kaufman, I.B. Lamster, 2000). The supernatants were stored at – 20◦C until the enzyme-linked immunosorbent assay (ELISA) analysis (Karla A., J. et al., 2019).

2.1 Ethical approval:

The protocol was approved by the Ethics committee, College of Dentistry, University of Baghdad (Reference number: : 946, Project number: 946624, Date: 14-10-2024, Appendix number: I). Before enrollment in the study, each patient was asked to sign an informed consent form after providing all information fully describing the nature and aims of the study . All procedures included in this study were in accordance with Helsinki declaration and its later amendments for human researches.

2.2 Statistical Analysis:

The study's findings were analysed using (SPSS version 21) (Chicago, USA, Illinois); For descriptive statistics, all data were expressed as frequency, percent, mean, standard deviation, and median. Prior to inferential analysis, Gaussian distribution of data was determined by using Shapiro-Wilk test which indicated that measurements obtained from ELISA were not normally distributed. Therefore, comparisons of multiple groups were performed by Kruskal-Wallis test and in case of significant results further intergroup comparison was carried out by using Bonferroni post hoc test. Periodontal parameters showed normal distribution; thus, multigroup comparisons were conducted by using ANOVA test followed by Tukey post hoc test when results were significant.

Diagnostic accuracy of biomarkers was determined by using receiver operating characteristic (ROC) curve and area under the curve (AUC). Level of significance was set at $p< 0.05$. All statistical analyses were conducted by using GraphPad Prism software (version 9, GraphPad Software, San Diego, CA, USA)

3. Results and Discussion:

Demographic characteristics of the study populations including age and sex distribution are illustrated in Table 3.1. according to age there is significant difference between the groups being compared. Distribution according to sex showed that female (n=48) represented 57% while male (n=36) represented 42% of the total sample (Table 3.1).

Table 1: Demographic data among groups

Vars.	H	G	PD	Statistics
Age^	28.679±4.372	34.714±8.793	45.200±10.142	0.000 Sig.
Gender^^	N (%)			
M	12 (42.86)	10(35.71)	14(46.67)	0.694 NS
F	16 (57.14)	17(64.29)	15(53.33)	

^=One Way ANOVA, ^^=Chi square

H:healthy control group, G: gingivitis group, PD: periodontitis group

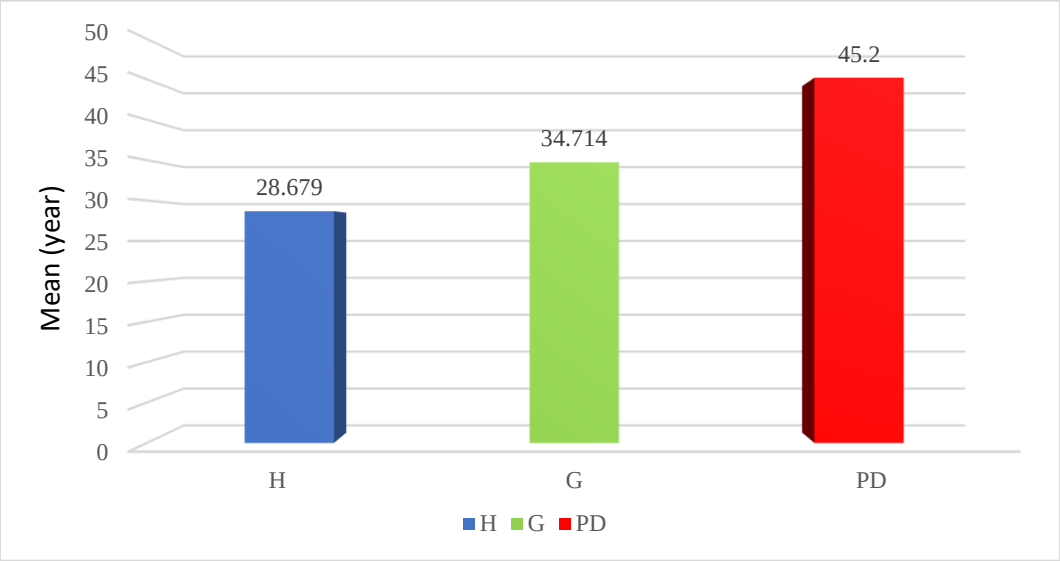

Figure 1: Bar Chart concerning frequency distribution of Demographical Characteristics variables of Age and Gender in studied groups

3.2 Normality test:

All studied variables were found to be normally distributed (parametric data) using Shapiro-Wilk at ($p>0.05$) (table 3.2).

Table 2: Normality test of studied variables

Vars.	H		G		PD	
	Statistic	P value	Statistic	P value	Statistic	P value
BOPP	0.941	0.115	0.955	0.264	0.939	0.103
GAL-3	0.930	0.068	0.934	0.071	0.966	0.482
PAD4	0.955	0.264	0.929	0.053	0.947	0.166

The results above show all studied variables are normally distributed among groups using the Shapiro-Wilk test at $p>0.05$.

Table 3 demonstrates the descriptive statistics for GAL-3 and PAD-4 analyzation among study groups. According to the table 3, the results demonstrated that the lowest mean value of GAL-3 and PAD-4 were in the control group, and it increased in both gingivitis and periodontitis groups with significant difference at ($p<0.05$) among the groups.

TABLE 3 saliva levels of biomarkers in study groups

Groups		Mean	±SD	Minimum	Maximum	F	P value	MPC using Games Howell
GAL-3	H	0.611	0.131	0.168	0.797	316.774	0.000 Sig.	H-G<0.001
	G	2.549	0.480	1.931	3.358			H-PD<0.001
	PD	3.029	0.432	2.024	3.802			G-PD==0.001
PAD-4	H	1.126	0.139	0.908	1.384	206.445	0.000 Sig.	H-G<0.001
	G	2.542	0.350	1.359	3.131			H-PD<0.001
	PD	3.568	0.685	1.559	5.285			G-PD==0.001

Multiple pairwise comparisons using Games-Howell post hoc test showed that all results were statistically significant (P<0.05) when comparing the control with other groups. Also, the comparison between patients' groups showed statistically significant (P<0.05) (figure 3.4).

The strong p-values imply that there was no significant association between BOP and the biomarkers (GAL3, PAD2, and PAD4) across all study groups. Particularly, in Groups H and G,

TABLE 4 Correlations of clinical and biochemical parameters

Groups		BOPP		PPD		CAL	
		r	p	r	p	r	p
H	GAL3	0.151	0.445				
	PAD4	0.320	0.096				
G	GAL3	0.109	0.581				
	PAD4	0.251	0.198				
PD	GAL3	0.185	0.346	0.109	0.581	0.292	0.131
	PAD4	0.172	0.380	0.024	0.904	0.116	0.555

r: Correlation coefficient. By Pearson's correlation test, significant difference at p<0.05.

Table 5 shows that in group H, GAL-3 exhibited no significant associations with PAD-4 (r = -0.033, p = 0.866) indicating a lack of association among these biomarkers.

In Group G, GAL-3 had a strong positive correlation with PAD-4 (r = 0.436, p = 0.020). indicating that a rise in GAL-3 is associated with an increase with PAD-4.

In Group PD, GAL-3 exhibited weak non statistically significant correlation with PAD-4 (r=0.217).

Table 5 Correlation between biomarkers among groups

Groups		PAD4	
		r	p
H	GAL3	-0.033	0.866
G	GAL3	0.436	0.020
PD	GAL3	0.217	0.268

r: Correlation coefficient. By Pearson's correlation test, significant difference at p<0.05

Table 6 illustrates the estimated area of cut-off between sensitivity and specificity by plotting sensitivity against the complement of specificity to examine this cut-off, known as the Receiver Operating Characteristic (ROC) curve. It also includes significant levels for testing the area under the curve against the guideline of fifty percent, with a 95% confidence interval for all probable combinations among patient groups and controls.

All biomarkers were found to have high sensitivity and specificity for separating healthy periodontal tissue from both gingivitis and periodontitis. The highest diagnostic accuracy for differentiating periodontal health from gingivitis was observed with GAL3, showing perfect sensitivity (1.000) and specificity (1.000) at an optimal cutoff value of 0.31. For the differentiation between periodontal health and periodontitis, GAL3 demonstrated excellent diagnostic performance with sensitivity (1.000) and specificity (0.999) at a cutoff value of 0.83, indicating its strong potential as a biomarker.

TABLE 6 Diagnostic value of salivary biomarkers

	Test Result Variable(s)	Area under curve (AUC)	P value	Sensitivity	Specificity	Optimal cutoff point
H-G	GAL-3	1.000	0.000	100	100	0.31
	PAD-4	0.999	0.000	100	100	0.919
H-PD	GAL-3	1.000	0.000	100	100	0.83
	PAD-4	1.000	0.000	100	100	0.919
G-PD	GAL-3	0.755	0.001	71.4	60.7	2.77
	PAD-4	0.948	0.000	92.9	72.1	2.80

H:healthy, G:gingivitis, PD:periodontitis, GAL-3:galectin-3, PAD-2:peptidylarginie deiminase-2, PAD-4:peptidylarginine deiminase-4.

The primary finding of our investigation was the elevated saliva concentrations of Gal-3 and PAD-4 in individuals with periodontal disease. Periodontal disorders arise from a complex interplay between subgingival dental biofilm and the body's immunological response. As dental plaque accumulation escalates, there is a corresponding increase in various defense cells within the connective tissues, notably neutrophils, plasma cells, and macrophages, along with the extracellular release of their destructive components, leading to inflammation and the emergence of the fundamental clinical manifestations of gingivitis and periodontitis.The current investigation revealed that salivary concentrations of galectin-3 were considerably elevated in group PD compared to group H, while group PD also exhibited higher levels than group G. Gal-3 participates in various phases of acute inflammation, encompassing neutrophil activation and adhesion, the death mechanism of neutrophils, and mast cell degranulation (Barut A., Z., et al., 2023).

Additionally, Gal-3 can bind to the integrins on the cell surface and induce inflammation by increasing the adhesion of the neutrophils and vascular endothelial cells. Gal-3 has several biological functions including cell aggregation, chemoattraction, and apoptosis (D.K. Hsu and F.T. Liu, 2000).

A previous study investigated the Gal-3 levels in the gingival crevicular fluid (GCF) in periodontitis, gingivitis, and healthy individuals and found that Gal-3 is a potential biomarker for gingival inflammation; periodontal therapy efficiently reduced the GCF Gal-3 levels (Karsiyaka Hendek, 2021). Study shows that in groups H and PD, GAL-3 exhibited no significant associations with PAD-4. Furthermore, GAL-3 demonstrated a strong positive connection with PAD-4 in G group. Concerning significant higher levels of GAL-3 in group PD than group G , also in group G than group H. This came in agreement with (Akkaya HÜ, 2022; Abdulmajeed et al., 2023). It has been

indicated that this protein is produced by chondrocytes, osteoblasts, and osteoclasts and plays important roles in bone metabolism (differentiation and/or activity of osteoblast and osteoclast) (Iacobini C et al., 2018). It has been demonstrated that galectin-3 was highly expressed in areas with intense cartilage and bone destruction and elevated levels of galectin 3 in the arthritis can induce chemokines/cytokines and reactive oxygen species production from fibroblasts and neutrophils and cause bone destruction (Li YJ et al., 2009). However, it has been also concluded that this molecule has a suppressive role in osteoclastogenesis (Li YJ et al., 2009). Considering the data above, difference between the salivary levels of galectin-3 in group PD and group G may be due to bone destruction in periodontitis lesions which is not observed in gingivitis lesions. This finding may support the possible association between galectin-3 and periodontal inflammation severity Higher galectin-3 levels in periodontitis may also be related to association between galectin-3 and Porphyromonas gingivalis (P.gingivalis) in this study. Miyauchi et al. showed that P.gingivalis Lipopolysaccharide stimulation increased galectin-3 expression in placental cells (Miyauchi M et al., 2018). Possible presence of P. gingivalis in periodontitis lesions in this present study may have increased galectin-3 production.

Considering the studies about PAD4 enzyme in periodontology field, expression of PAD4 at the protein and mRNA levels was detected in periodontal tissue (Harvey GP et al., 2018).

In this present study, which is the first to study levels of PAD-4 in saliva of health and diseased periodontium, salivary PAD4 total levels was significantly higher in the group PD comparing group G, also higher significantly in G than H. This came in agreement with Nesse et al. that demonstrated that presence of elevated citrullinated protein (80%) in periodontitis connective tissue was found compared with control connective tissue (33%) (Nesse W et al., 2012).

Citrullination also plays a role in physiological processes such as skin keratinization (Baka Z et al., 2012) and it was reported that it is a physiological process in periodontal epithelium.18 This information and possible subclinical gingivitis condition may explain why PAD4 was detected in group HP.

In this study, the results disagree with Akkaya.H.U (Akkaya HÜ et al., 2022) in which evaluate GCF levels of PAD-4, showed that PAD4 levels were similar in periodontitis group and gingivitis group even if the group PD has slightly higher levels than the group G. This situation can be explained with similar sampling sites for both groups in terms of inflammation degree (BOP (+), GI = 2 for sampling sites in both group). While there was a difference between periodontitis group and health group in terms of PAD4 levels, there was no difference between gingivitis group and healthy group. This situation can be explained as follows, The PAD4 levels were investigated in GCF by paper strip method. The paper strips are in contact with the epithelium in this method. The fact that citrullination is physiological process in the epithelium may have led to no difference between group HP and group G.

Engström et al. found no difference for PAD4 levels in the gingival epithelium of patients with periodontitis and periodontal healthy individuals. They explained this situation with the fact that the citrullination process is a physiological process in the epithelium (Engstrom M et al., 2018).

Other studies came in agreement with our results which show that human PADs are calcium-dependent enzymes; the binding of PAD enzyme with calcium promotes bioactive conformation and increases PAD activity 10,000 times.(Baka Z et al., 2012)

It was shown that calcium increased in diseased root surface adjacent to the periodontal pocket (Selvig KA and Zander H., 1962). This information may explain the higher PAD4 levels in periodontitis group compared with healthy group.

Conclusions:

Within the limitations of this study, increased salivary Gal-3 and PAD-4 levels were found in periodontitis and gingivitis patients. These results suggest that salivary Gal-3 and PAD-4 may be potential biomarkers for periodontal diseases. These molecules can be detected in the saliva of patients with periodontal disease and may be effective in the early diagnosis and treatment of these diseases.

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Conflicts of Interest Statement……

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