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Dentistry Section

Evaluation of Lipocalin-2 Levels in Gingival Crevicular Fluid and Serum of Periodontitis Patients: A Cross-sectional Study

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ABSTRACT

Introduction: Periodontitis is a chronic inflammatory disease affecting the supporting structures of teeth, and directly challenges the immune response, presented in elevation of many inflammatory mediators and cytokines. Lipocalin-2 (LCN-2), which belongs to the lipocalin family, plays a role in numerous pathophysiological processes. Additionally, it acts as an inflammatory mediator in several inflammatory conditions and may possess antimicrobial characteristics.

Aim: To compare the levels of LCN-2 in the serum and Gingival Crevicular Fluid (GCF) of patients with different stages of periodontitis and periodontally healthy individuals in Saudi Arabia.

Materials and Methods: The present cross-sectional study was conducted on patients seeking dental management at the Dental Teaching Hospital, College of Dental Medicine, Umm Al-Qura University, Makkah, Saudi Arabia between February and November 2024. Forty systemically healthy non-smoking individuals were categorised into four groups: periodontally healthy individuals and those with stages I, II, and III generalised periodontitis (n=10 per group). The clinical periodontal parameters were documented, and GCF and serum samples were obtained from all the participants. The GCF and serum LCN-2 levels were measured using an Enzyme-Linked Immunosorbent Assay (ELISA). One-way Analysis of Variance (ANOVA) and Tukey's multiple-comparison tests were used to assess the results, and a p-value <0.05 was deemed statistically significant.

Results: The clinical periodontal parameters revealed significant differences in clinical attachment loss among the periodontitis stages (p<0.001). Patients at each of these stages had significantly higher GCF LCN-2 levels compared to the healthy individuals (p<0.05), although the differences between the stages were not statistically significant. However, only the stage II periodontitis patients exhibited significantly higher serum LCN-2 levels than the stage I patients and healthy individuals (p<0.05).

Conclusion: Significantly higher LCN-2 level in GCF was found in patients with different stages of periodontitis compared with the healthy controls, indicating that LCN-2 might be considered a local inflammatory marker in periodontitis.

Keywords: Biomarkers, Immunologic factors, Inflammation, Neutrophil gelatinase-associated lipocalin, Periodontal diseases

INTRODUCTION

Periodontitis still remains a global public health problem, with an estimated nearly 62% of adults affected [1]. It is defined by clinical destruction of periodontal attachment and alveolar bone as a result of bacterial infection. This elicits an immune reaction that creates a host-modulated response [2], which causes the production of pro-inflammatory cytokines and other inflammatory mediators [3].

At present, periodontitis is evaluated based on clinical periodontal parameters and the degree of radiographic alveolar bone loss [4]. While these parameters give an indication of the present stage of the disease, they are inadequate in predicting the risk or progression of the disease [5]. Novel methods to identify various inflammatory mediators in GCF, saliva, as well as serum, are necessary to provide more accurate indications of risk, progression, and therapeutic targets [6-8] and can be utilized to improve the precision of the diagnosis of early periodontitis and severity [9].

Lipocalin-2 (LCN-2)/neutrophil gelatinase-associated lipocalin (NGAL) is a 25-kD glycoprotein which is implicated in several biological processes, such as transport of fatty acids, programmed cell death, and regulation of inflammation [10]. The LCN-2 contributes to iron depletion by binding to bacterial ferric siderophores, thereby suppressing the growth of iron-oxidizing bacteria through iron deprivation [11]. LCN-2 is considered an inflammatory mediator, and its expression is induced by lipopolysaccharide, tumour necrosis factor-alpha, and interleukin (IL)-1β [10,12]. It plays a role in inflammatory diseases such as inflammatory bowel disease, severe acute pancreatitis, and arthritis [13-16]. In addition to its role in inflammation, it suppresses the adherence of *Porphyromonas gingivalis* to oral epithelial cells and inhibits the expression of IL-8 and macrophage inflammatory protein-1α (MIP-1α) in periodontal tissue cells and neutrophil-like cells (HL-60) [17,18]. These findings suggested that LCN-2 may play a role in inflammatory downregulation and antibacterial activity.

Neutrophils are the primary source of LCN-2 [19]. LCN-2 has been found to be an adjunctive biochemical marker for tracking the progression and treatment outcomes of periodontal conditions in GCF and saliva [20]. In addition, a link has been found between serum and salivary LCN-2 levels and the severity of periodontal disease [21]. Indeed, numerous studies have linked periodontal diseases to higher LCN-2 levels [20-25]. However, although LCN-2 levels in gingivitis and periodontitis had been investigated, the literature on LCN-2 levels in different stages of periodontitis is scarce. Nevertheless, GCF LCN-2 levels have been linked with clinical parameters and could be helpful as non-invasive assessment methods for periodontitis [22]. Therefore, the objective of the present study was to evaluate the GCF and serum LCN-2 levels in patients with various stages of periodontitis and periodontally healthy individuals in Saudi Arabia.

MATERIALS AND METHODS

The present cross-sectional study was conducted on patients seeking dental management at the Dental Teaching Hospital, College of Dental Medicine, Umm Al-Qura University, Makkah, Saudi Arabia between February and November 2024. Ethical

approval (Registration No: HAPO-02-K-012-2022-03-1040) was obtained, and all participants were informed about the research process and approaches before obtaining their written informed consent.

Inclusion and Exclusion criteria: To be eligible, each participant must be systemically healthy, non smokers, and have at least 20 permanent teeth. Patients with systemic disorders, those with pre existing medical conditions, those who were pregnant or nursing, those who had received antibiotics or steroidal or non steroidal anti-inflammatory drugs during the previous three months, and those who had received periodontal therapy within the previous six months were excluded.

Sample size calculation: The sample size was calculated using G*Power (version 3.1.9.7) for a one-way ANOVA comparing four independent groups: healthy controls and periodontitis stages I, II, and III. Based on a minimal clinically significant difference and a standard deviation from a previous study [25], the effect size was estimated as 0.781. Using a significance level of 0.05 and a power of 95%, the required total sample size was 36 participants, with 9 participants per group.

Study Procedure

Periodontal assessment: A comprehensive periodontal examination and radiographic evaluation of the level of alveolar bone loss were performed for each participant. The periodontal examination included the following parameters: Plaque Index (PI), Bleeding Index (BI) [26,27], Pocket Depth (PD), and Clinical Attachment Loss (CAL) [28]. The PI and BI scores were recorded at four surfaces per tooth (mesial, distal, buccal, and palatal/lingual). In addition, PD and CAL were measured at six surfaces per tooth (mesiobuccal, midbuccal, distobuccal, mesiopalatal/lingual, midpalatal/lingual, and distopalatal/lingual) and recorded to the nearest millimeter using a sterile periodontal probe (William's, Hu-Friedy Manufacturing Corp., Chicago, IL, USA).

The included participants were classified into four groups: the healthy individuals (n=10) were defined as those having a PD ≤3 mm, BI <10%, no CAL, and no radiographic bone loss [29]. In contrast, the generalised periodontitis patients (n=30) had a BI ≥10%. They were classified as follows: stage I (n=10), with a PD ≤4 mm and a CAL of 1-2 mm; stage II (n=10), with a PD ≤5 mm and a CAL of 3-4 mm; and stage III (n=10), with a PD ≥6 mm and a CAL ≥5 mm. The percentages of periodontal bone loss were <15% in stage I, 15-33% in stage II, and >33% in stage III periodontitis [30].

Samples collection: Blood was collected into a 5-mL tube (Thermo Fisher Scientific Inc.) and allowed to clot at the room temperature degree for 30 min, then centrifuged at 3,000 rpm for 10 minutes. After that, the serum was transported into a 1.5-mL Eppendorf tube. GCF samples were collected after the periodontal examination from isolated sites in the teeth in the upper jaw to minimise the risk of contamination with saliva. Supragingival plaque and calculus were carefully removed without disturbing the gingival crevice. Afterward, a micropipette capillary tube (Merck KGaA, Sigma-Aldrich, Darmstadt, Germany) was inserted into the gingival crevice and moved gently to facilitate fluid flow into the tube, providing collection of a 5 µL standardised GCF sample. Each sample was transferred into a sterile 1.5-mL Eppendorf tube containing 200 µL of phosphate-buffered saline at a 40-fold dilution. All the GCF and serum samples were kept in a freezer at -80°C until use.

LCN-2 immunoassay: The LCN-2 levels were quantified in the serum and GCF using enzyme-linked immunosorbent assay (Invitrogen, Thermo Fisher Scientific), following the manufacturer's instructions. The serum samples were diluted 30-fold before they were added to the ELISA plate coated with LCN-2-specific antibodies. Then, the absorbance values were measured at 450 nm using a spectrophotometric ELISA microplate reader (Spectro Star; BMG LABTECH, Offenburg, Germany). Duplicate measurements were conducted to ensure accuracy, and the total amounts of LCN-2 were calculated and expressed in ng/mL.

STATISTICAL ANALYSIS

The data were displayed as means±standard errors of the mean (SE) and analysed using GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA) and Microsoft Excel. The differences between the pairs of groups in the clinical parameters and the LCN-2 levels were calculated and compared at different periodontitis stages and in healthy subjects by one-way ANOVA and Tukey's multiple-comparison tests. A p-value<0.05 was deemed statistically significant.

RESULTS

A total of 40 individuals between the ages of 20 and 60 years old with mean±SD (33.30±11.05), they were selected as study participants. Among them, 23 were female (57.5%), and 17 were male (42.5%). The participants were divided based on their periodontal diagnosis into four groups: healthy, stage I, stage II, and stage III periodontitis (n=10 per group).

The mean±SE of periodontal clinical parameters in healthy and various stages of periodontitis are shown in [Table/Fig-1]. All clinical periodontal parameters showed significant differences between healthy and various stages of periodontitis (p<0.001). PI significantly increased from stage I to stage III (p=0.019), but there were no significant changes between stages I and II and between stages II and III. BI and PD did not differ significantly across the stages (p>0.05), in contrast, CAL exhibited significant differences across the stages (p<0.001), reflecting the severity of periodontitis [Table/Fig-2].

The mean±SE of GCF and serum LCN-2 levels in healthy and various stages of periodontitis are shown in [Table/Fig-3]. The

Parameters	Healthy	Stage I	Stage II	Stage III
PI (%)	8.89±1.50	36.66±6.59	52.23±8.13	64.14±4.68
BI (%)	5.54±1.47	48.22±8.19	50.54±8.90	45.87±4.96
PD (mm)	1.89±0.07	2.51±0.17	2.95±0.28	3.20±0.24
CAL (mm)	0.14±0.06	1.90±0.12	3.34±0.35	5.81±0.50

[Table/Fig-1]: Clinical periodontal parameters (Mean±SE) in healthy individuals and patients with different stages of periodontitis.
Notes: SE: standard error; PI: plaque index; BI: bleeding index; PD: pocket depth; CAL: clinical attachment loss.

Parameters	Comparison of Mean±SE		p
PI (%)	H (8.89±1.50)	S I (36.66±6.59)	<0.001*
	H (8.89±1.50)	S II (52.23±8.13)	<0.001*
	H (8.89±1.50)	S III (64.14±4.68)	<0.001*
	S I (36.66±6.59)	S II (52.23±8.13)	0.240
	S I (36.66±6.59)	S III (64.14±4.68)	0.019*
	S II (52.23±8.13)	S III (64.14±4.68)	0.424
BI (%)	H (5.54±1.47)	S I (48.22±8.19)	0.004*
	H (5.54±1.47)	S II (50.54±8.90)	<0.001*
	H (5.54±1.47)	S III (45.87±4.96)	<0.001*
	S I (48.22±8.19)	S II (50.54±8.90)	0.974
	S I (48.22±8.19)	S III (45.87±4.96)	0.973
	S II (50.54±8.90)	S III (45.87±4.96)	0.900
PD (mm)	H (1.89±0.07)	S I (2.51±0.17)	0.014*
	H (1.89±0.07)	S II (2.95±0.28)	0.005*
	H (1.89±0.07)	S III (3.20±0.24)	<0.001*
	S I (2.51±0.17)	S II (2.95±0.28)	0.390
	S I (2.51±0.17)	S III (3.20±0.24)	0.121
	S II (2.95±0.28)	S III (3.20±0.24)	0.756

CAL (mm)	H (0.14±0.06)	S I (1.90±0.12)	0.004*
	H (0.14±0.06)	S II (3.34±0.35)	<0.001*
	H (0.14±0.06)	S III (5.81±0.50)	<0.001*
	S I (1.90±0.12)	S II (3.34±0.35)	<0.001*
	S I (1.90±0.12)	S III (5.81±0.50)	<0.001*
	S II (3.34±0.35)	S III (5.81±0.50)	<0.001*

[Table/Fig-2]: Comparisons of the periodontal clinical parameters between healthy and different stages of periodontitis.
Notes: SE: standard error; PI: plaque index; BI: bleeding index; PD: pocket depth; CAL: clinical attachment loss; H: healthy; SI: stage I periodontitis; SII: stage II periodontitis; SIII: stage III periodontitis. *Indicate significant p-values.

Parameters	Healthy	Stage I	Stage II	Stage III
GCF LCN-2 (ng/mL)	3.243±1.50	16.88±2.27	28.15±4.33	16.71±3.32
Serum LCN-2 (ng/mL)	16.58±1.39	14.55±2.15	26.36±2.88	17.26±2.74

[Table/Fig-3]: The mean±SE of GCF and serum LCN-2 levels in healthy and various stages of periodontitis.
Notes: SE: standard error; GCF: gingival crevicular fluid.

data analysis revealed significant differences in GCF LCN-2 levels between the healthy individuals and the patients in different stages of periodontitis ($p<0.05$), but the differences between the stages of periodontitis were not significant ($p>0.05$), as shown in [Table/Fig-4]. There was no significant difference in serum LCN-2 levels between the healthy individuals and the stages I and III periodontitis patients. However, the serum LCN-2 levels of the stage II patients significantly differed from those of the stage I patients and the healthy individuals [Table/Fig-4].

Parameters	Comparison of Mean±SE		p
GCF LCN-2 (ng/mL)	H (3.243±1.50)	S I (16.88±2.27)	0.011*
	H (3.243±1.50)	S II (28.15±4.33)	<0.001*
	H (3.243±1.50)	S III (16.71±3.32)	0.017*
	S I (16.88±2.27)	S II (28.15±4.33)	0.054
	S I (16.88±2.27)	S III (16.71±3.32)	0.999
	S II (28.15±4.33)	S III (16.71±3.32)	0.061
Serum LCN-2 (ng/mL)	H (16.58±1.39)	S I (14.55±2.15)	0.948
	H (16.58±1.39)	S II (26.36±2.88)	0.049*
	H (16.58±1.39)	S III (17.26±2.74)	0.997
	S I (14.55±2.15)	S II (26.36±2.88)	0.013*
	S I (14.55±2.15)	S III (17.26±2.74)	0.877
	S II (26.36±2.88)	S III (17.26±2.74)	0.058

[Table/Fig-4]: Comparison of GCF and serum LCN-2 levels between healthy and different stages of periodontitis.
Notes: LCN-2: lipocalin-2; SE: standard error; H: healthy; SI: stage I periodontitis; SII: stage II periodontitis; SIII: stage III periodontitis. *Indicate significant p-values.

DISCUSSION

The present study aimed to assess the clinical periodontal parameters and LCN-2 levels in GCF and serum among patients with various stages of periodontitis and healthy individuals in Saudi Arabia. In the present study, the clinical periodontal parameters, which indicate the severity of periodontitis, demonstrated a notable increase in PI from stage I to stage III and significant changes in CAL across the periodontitis stages. These results are consistent with those of other studies that examined the periodontal status at various stages of the disease [31,32].

The current study investigated the local (GCF) and systemic (serum) levels of LCN-2 in periodontally healthy controls and stages I, II, and III periodontitis patients. The GCF LCN-2 levels were significantly higher in the periodontitis groups than in the healthy control group. These results were consistent with several studies that reported high LCN-2 levels in periodontitis [20-25], suggesting that LCN-2 may significantly contribute to the disease's pathophysiology by its involvement in neutrophil-mediated inflammation and bone remodelling facilitated through osteoblast activity [19]. For instance, Pradeep AR et al. found significantly elevated GCF LCN-2 concentration in obese patients with chronic periodontitis as compared to periodontally healthy subjects and non-obese chronic periodontitis patients [25]. Similarly, Alkayali MF et al. introduced LCN-2 as a potential biomarker of periodontal disease [33]. Tsuchida S et al. also confirmed a significant rise in LCN-2 concentration in periodontitis patients compared to healthy controls, which indicates the value of LCN-2 levels in detecting periodontal disease [34]. Elevated GCF LCN-2 levels in stage II compared with other periodontitis stages were also determined in the present study. Nevertheless, the lack of significance of the differences was likely related to the small number of periodontitis participants in this sample.

The LCN-2 levels in the serum of stage II periodontitis were significantly different from those of healthy and stage I. These results were also in line with the results of Tan A et al. [21], who reported that the level of LCN-2 in serum was conspicuously increased in periodontitis patients relative to healthy subjects. However, in the current study, there was a nonsignificant difference in the serum LCN-2 levels of the stages I and III patients from those of healthy individuals. This outcome is contrary to the observations of Tan A et al. and Hamdi AQ et al. [21, 24] and may be related to different systemic variances that might have influenced the LCN-2 serum levels.

In the present study, it is noted that the GCF and serum levels in stage II were higher than those in stage III and the other groups. This raises the question of why the LCN-2 levels decreased in stage III despite the disease's advanced stage. Previous studies suggested that LCN-2 might exhibit not only inflammatory responses but also biological effects, such as antibacterial properties, and might downregulate some immunologic cytokines, such as IL-8 and MIP-1α [17,18,35], which might explain the increase in its levels in stage II periodontitis patients.

Limitation(s)

There are some limitations to the present study that should be mentioned. Firstly, the small population may have contributed to the lack of significant differences between some of the periodontitis stages. Second, stage IV periodontitis was excluded from this study because of its low patient rate diagnosed at this stage. In addition, the role of other variables, such as age, gender, and body mass index were not addressed in the present study and may be interesting aspects that should be investigated in future studies in order to clarify the role of LCN-2 in periodontitis. Nevertheless, the main strength of the study is the evidence added to the clarification on inflammatory biomarkers and their relationship with the periodontal disease process.

CONCLUSION(S)

In the present study, the LCN-2 level in GCF was found significantly higher in patients with different stages of periodontitis compared with the healthy controls, indicating that LCN-2 might be considered a local inflammatory marker in periodontitis. More comprehensive studies on large populations, including more variables through various stages of periodontitis, are necessary to clearly elucidate the role of LCN-2 in the progression of periodontitis.

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