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Diagnostic potential of salivary interleukin-17, RANKL, and OPG to differentiate between periodontal health and disease and discriminate stable and unstable periodontitis: A case-control study

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Abstract

Background and Aims: Limitations of the conventional diagnostic techniques urged researchers to seek novel methods to predict, diagnose, and monitor periodontal disease. Use of the biomarkers available in oral fluids could be a revolutionary surrogate for the manual probing/diagnostic radiograph. Several salivary biomarkers have the potential to accurately discriminate periodontal health and disease. This study aimed to determine the diagnostic sensitivity and specificity of salivary interleukin (IL)-17, receptor activator of nuclear factor-κB ligand (RANKL), osteoprotegerin (OPG), RANKL/OPG for differentiating (1) periodontal health from disease and (2) stable and unstable periodontitis.

Methods: Participants with periodontitis (n = 50) and gingivitis (n = 25), both diseases represented the cases, and subjects with healthy periodontium (n = 15) as a control were recruited for this study. Periodontitis cases were further equally subdivided into stable and unstable. Whole unstimulated salivary sample were collected from all participants. Periodontal parameters including bleeding on probing, probing pocket depth, clinical attachment loss, and number of missing teeth were recorded. The protein levels of salivary IL-17, RANKL, and OPG were determined by using enzyme-linked immunosorbent assays technique.

Results: Salivary IL-17, OPG, RANKL, and RANKL/OPG showed high sensitivity and specificity to differentiate periodontal health from gingivitis and periodontitis. Similar pattern was observed in discriminating stable and unstable periodontitis. Salivary IL-17 and RANKL showed a good accuracy to differentiate gingivitis from periodontitis. However, OPG and RANKL/OPG did not exhibit enough sensitivity and specificity to differentiate the latter conditions.

Conclusion: Salivary IL-17, RANKL, OPG, and RANKL/OPG system are potential candidates for differentiating periodontal health and disease and discriminate stable and unstable periodontitis.

KEYWORDS
biomarkers, interleukin-17, osteoprotegerin, periodontal diseases, RANKL, saliva

1 | INTRODUCTION

New domains for periodontitis diagnosis were introduced in the 2018 classification of periodontal and peri-implant diseases.[1] Notably, patients with periodontitis even when successfully treated still diagnosed with periodontitis. The pathologically-reduced periodontium due to the previous disease activity is more vulnerable to destruction than healthy intact tissue. To differentiate state of health for intact and reduced periodontium, the latter was called stable periodontitis.[2] The case definition and diagnostic criteria of stable periodontitis was clearly defined by the latest classification system.[2]

Dysbiotic dental plaque biofilm is the main driver for the initiation and progression of periodontitis.[3] The immune response to the presence of pathogenic periodontopathogens leads to the buildup of immune cells in subjacent periodontal tissues.[4] Consequently, a range of proinflammatory and inflammatory cytokines are released causing tissue damage and bone resorption.[5] The fact that the concentrations of these cytokines are remarkably increased during periodontal disease versus healthy state encouraged researchers to use them as biomarkers available in oral fluids for prediction, diagnosis, and monitoring periodontal disease.[6–9]

Interleukin (IL)-17, the distinctive cytokine of T-helper cells (Th) 17, is essential for immunological inflammatory disorders such rheumatoid arthritis and periodontitis.[10] Previous studies have demonstrated that periodontitis lesions exhibit higher levels of IL-17 than healthy control tissue,[11] and its level is positively correlated with the periodontitis-associated destruction.[12–14] Data from experimental animal models showed that mice lacking Th17 cells experienced decreased bone loss and inflammation during periodontitis.[15,16] This suggests that IL-17 is likely contributing to the loss of alveolar bone through increasing the expression of receptor activator of nuclear factor-κB ligand (RANKL) in mesenchymal cells.[4]

RANKL is a member of the TNF superfamily which has a pivotal regulatory role in osteoclastogenesis by directly acting on osteoclast fusion, differentiation, activation, and persistence.[17] Clinical studies have shown a substantial correlation between the severity of periodontitis and RANKL concentration.[18] Action of RANKL is balanced by osteoprotegerin (OPG) which is a critical osteoprotective factor maintaining the homeostasis of bones by acting as a RANKL-decoy receptor primarily expressed by osteoblast lineage cells.[17,19–21] It has been demonstrated that oral bacteria and osteoclast-derived proteases degrade OPG and stimulate osteoclastogenesis in vitro.[22] In addition, OPG-deficient mice spontaneously experienced significant alveolar bone loss.[23] Potent virulence factors of Gram-negative bacteria for example, lipopolysaccharides and inflammatory cytokines produced by immune cells such as IL-17 and TNF-α, increase the RANKL/OPG ratio in osteoblastic cells and periodontal ligament cells.[24] These studies have suggested that periodontal bone loss is the net result of upregulation of RANKL and the downregulation/degradation of OPG.

This study aimed to determine diagnostic sensitivity and specificity of salivary IL-17, RANKL, OPG, RANKL/OPG for differentiating (1) periodontal health from disease and (2) stable and unstable periodontitis.

2 | MATERIALS AND METHODS

2.1 | Study design

This prospective case-control study was conducted at the Teaching Clinics of the Department of Periodontics, College of Dentistry, University of Baghdad from April to July 2022. The study obtained ethical approval from the Ethics committee, College of Dentistry, University of Baghdad (Ref. 532, 17/04/2022, Project # 532622). All participants signed a consent form after receiving detailed clarification about the study.

The participants were consecutively recruited and categorized into healthy periodontium (Ctrl), gingivitis, stable periodontitis, and unstable periodontitis. Ctrl was defined when bleeding on probing (BOP) <10%, periodontal probing depth (PPD) ≤3 mm, intact periodontium (no probing attachment loss).[25] Gingivitis diagnosed when BOP > 10%, PPD ≤ 3 mm, intact periodontium.[25] Case definition of stable periodontitis was BOP < 10%, PPD ≤ 4 mm, and no BOP at 4 mm sites.[1] While subjects with unstable periodontitis should exhibit PPD ≥6 or ≥4 mm with BOP.[1] The latter three groups represented the cases for this study.

2.2 | Inclusion and exclusion criteria

Individuals with no history of any systemic disease, nonsmoker, have more than 20 teeth, and willing to participate were included in the study. Subjects having dental implant(s), suffering from systemic or inflammatory diseases such as liver and/or kidney dysfunction, Crohn's disease, previous history of organ transplant or cancer therapy, or had any cardiovascular or renovascular disease or disorder, smoker individuals, and alcoholic were excluded. Individuals currently under active periodontal or orthodontic treatment, having periapical inflammation, receiving antibiotic treatment or immunosuppressant medication within the last 3 months, long-term medication with contraceptive and similar hormone compounds, salivary gland diseases, pregnant or lactating mothers, any oral lesion not related to periodontitis were also excluded.

2.3 | Periodontal parameters and clinical examination

Full periodontal charting including BOP, PPD, clinical attachment level, and number of missing teeth were recorded for each participant. Six sites per tooth were examined using a periodontal probe (Michigan O Probe; Osung USA) to record clinical periodontal parameters excluding wisdom teeth.

2.4 | Collection of salivary samples

Participants were refrained from eating or drinking for 1–2 h before the saliva collection. First, the participants were asked to wash their mouths thoroughly with water to remove any debris or contaminating material before collection. Then whole unstimulated saliva was collected into sterile test tubes.[26] Collected samples were centrifuged (80-1 Electronic Centrifuge) at 1000 rpm for 15 min to separate cellular debris from the salivary supernatants. A micropipette was used to aspirate a 500 µL of the clear salivary supernatants into a plastic Eppendorf tube containing 50 µL protease inhibitor enzyme solution. The Eppendorf tube were labeled and frozen at −20°C until analysis.

2.5 | Enzyme-linked immunosorbent assays (ELISA)

The samples were thawed and left for a few minutes to reach room temperature. Commercially available ELISA kits (MyBioSource) were used for measuring protein levels of salivary IL-17, RANKL, and OPG. The procedure was conducted following the manufacturer's instructions for each kit. Optical density (OD) was measured with a Microtiter plate reader (HumanReader HS; HUMAN Society for Biochemica and Diagnostica mbH). All OD readings were exported to spread sheets and converted into concentrations using linear regression equation specific for each biomarker.

2.6 | Statistical analysis

Descriptive statistics including mean, standard deviation, and median were used for the continuous data while frequency and percentage were used for describing categorial variables. Data distribution was checked by using Shapiro–Wilk test. For parametric continuous variables, analysis of variance test followed by post hoc analysis was used. Correlation between concentrations of salivary biomarkers was performed by Pearson's correlation test. Sensitivity and specificity of the biomarkers was investigated by using receiver operating characteristic (ROC) curve and area under the curve (AUC). All analyses were performed by using GraphPad prism (version 9.0) software. Differences were considered statistically significant when (probability) $p < 0.05$.

3 | RESULTS

A total of 90 participants were recruited in this study who were divided to four groups, namely; healthy periodontium (Ctrl, $n = 15$), gingivitis ($n = 25$), stable, and unstable periodontitis ($n = 25$ each) (Table 1). Frequency distribution of the participants according to sex, mean age of the participants, and clinical periodontal parameters in each group are illustrated in Table 1.

Biochemical analyses showed that the concentration of salivary IL-17 and RANKL in gingivitis and periodontitis groups were

TABLE 1 Demographic and clinical periodontal parameters of the study groups.

	Ctrl	Gingivitis	Stable periodontitis	Unstable periodontitis	Total
Sex (*n*, %)					
Male	6–6.7	13–14.4	15–16.7	13–14.4	47–52.2
Female	9–10.0	12–13.3	10–11.1	12–13.3	43–47.8
Total	15–16.7	25–27.8	25–27.8	25–27.8	90–100
Age[a]	23.7 ± 3.2	23.8 ± 5.6	52.2 ± 10.6	46.1 ± 9.9	37.8 ± 15.3
Clinical parameters					
BOP[a]	3.9 ± 1.4	52.9 ± 11.6	8.3 ± 1.4	59.6 ± 14.9	
PPD[a]	-	-	2.7 ± 1.0	3.9 ± 1.8	
CAL[a]	-	-	2.4 ± 0.7	3.8 ± 0.8	
Missing teeth[a]	-	0.7 ± 0.8	2.9 ± 2.2	4.1 ± 2.7	

Abbreviations: BOP, bleeding on probing; CAL, clinical attachment level; Ctrl, control (healthy periodontium); PPD, probing pocket depth.

[a]Mean ± SD.

significantly higher than Ctrl. Levels of IL-17 and RANKL in periodontitis cases were significantly higher than gingivitis group (Figure 1). However, level of salivary OPG and RANKL/OPG ratio were only significantly different between Ctrl group and diseased groups that is, gingivitis and periodontitis with no significant difference between the two latter (Figure 1). Concentrations of all biomarkers and RANKL/OPG ratio in salivary samples of unstable periodontitis cases were significantly higher than stable periodontitis (Figure 1).

Diagnostic sensitivity and specificity of the selected salivary biomarkers were determined by ROC curve and AUC (Figure 2). All salivary biomarkers and their ratio showed high sensitivity and specificity to differentiate periodontal health from gingivitis (AUC ranged between 0.875 and 0.993) and periodontitis (AUC ranged from 0.879 to 0.977). Similarly, these biomarkers showed the same pattern in differentiating periodontal health on a reduced periodontium that is, stable periodontitis from unstable periodontitis (AUC range from 0.889 to 0.964). Salivary IL-17 and RANKL showed

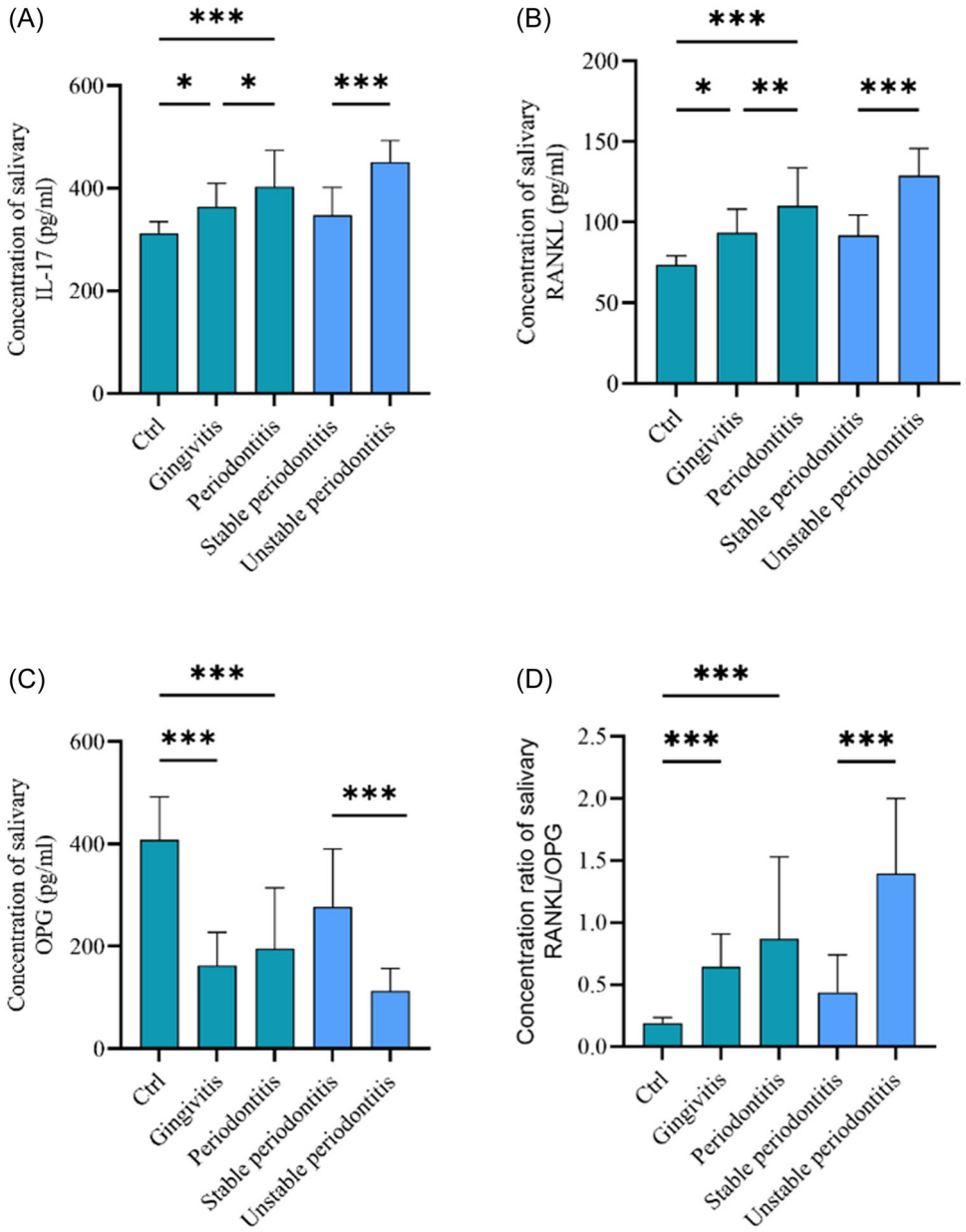

FIGURE 1 Salivary concentrations of IL-17, RANKL, OPG, and RANKL/OPG ratio. The levels of (A) IL-17 and (B) RANKL were significantly higher in participants with periodontitis than gingivitis and Ctrl (healthy periodontium). The same biomarkers were significantly higher in gingivitis group than the Ctrl. (C) Concentration of salivary OPG was significantly higher in Ctrl group than gingivitis and periodontitis counterparts; however, no significant difference was observed between the two latter. (D) Ratio of RANKL/OPG system did not show any significant difference between salivary samples of participants with gingivitis and periodontitis but both groups were significantly higher than Ctrl. All biomarkers (A–D) were significantly different between stable and unstable periodontitis. *p* Value *0.03, **0.002, ***>0.001. IL, interleukin; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor-κB ligand.

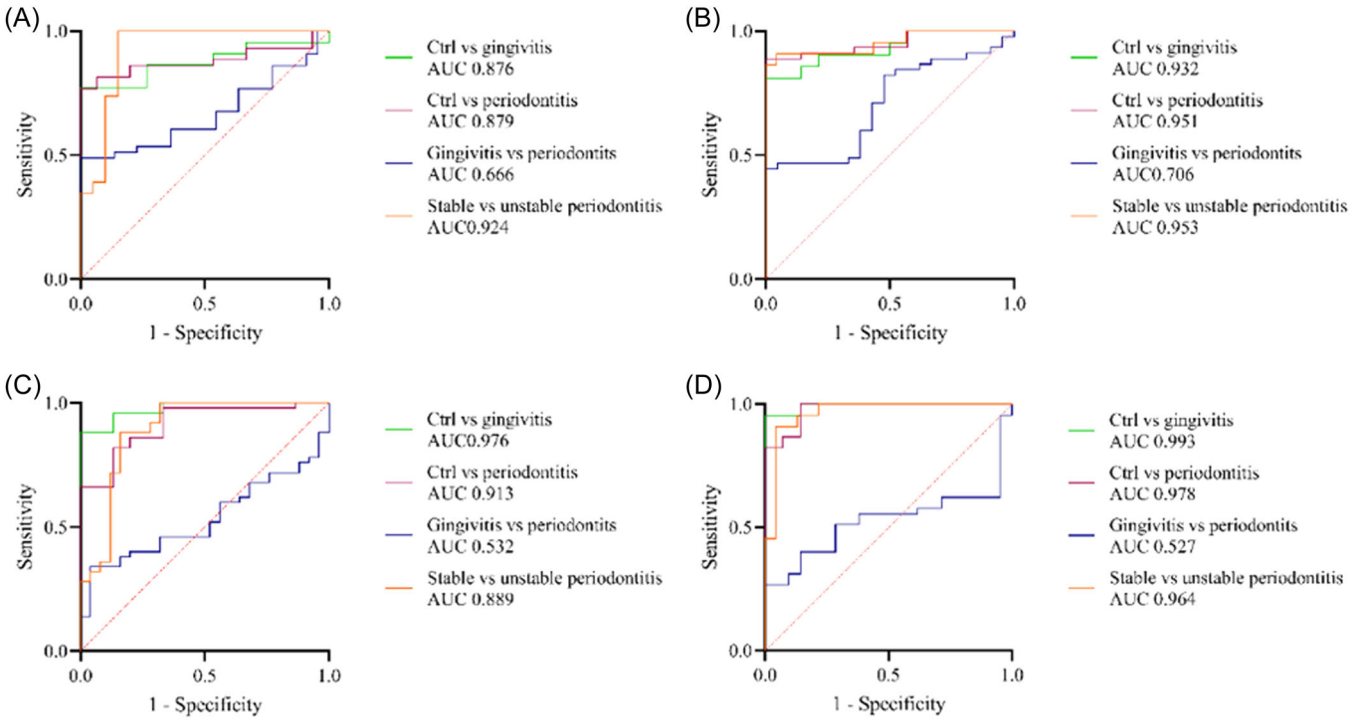

FIGURE 2 Receiver operating characteristic of salivary biomarkers (A) IL-17, (B) RANKL, (C) OPG, and (D) RANKL/OPG. All biomarkers showed high accuracy to distinguish periodontal health from periodontitis and gingivitis. IL-17 showed the lowest value to differentiate Ctrl (healthy periodontium) from gingivitis (AUC: 0.876) and periodontitis (AUC: 0.879). While ratio of RANKL/OPG system showed the highest potential to differentiate Ctrl from gingivitis (AUC: 0.993) and periodontitis (AUC: 0.978). Additionally, salivary biomarkers showed high accuracy to differentiate between stable and unstable periodontitis with AUC ranging from 0.889 to 0.964. Both IL-17 and RANKL showed moderately good accuracy to discriminate between gingivitis and periodontitis (AUC: 0.666 and 0.706, respectively). AUC, area under the curve; IL, interleukin; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor-κB ligand.

diagnostic potential to differentiate between gingivitis and periodontitis (AUC: 0.666 and 0.706, respectively). While salivary OPG and RANKL/OPG lack the accuracy to discriminate gingivitis from periodontitis. Sensitivity, specificity, and proposed cut-off concentrations of each biomarker and RANKL/OPG to differentiate periodontal health, on intact or stable periodontium, from periodontal disease are shown in Table 2.

Correlation analyses showed a positive and significant relation ($r = 0.545$, $p < 0.001$) between concentrations of salivary IL-17 and RANKL. On contrary, level of salivary OPG was significantly and negatively associated with IL-17 ($r = -0.364$, $p = 0.02$) and RANKL (−0.496, $p = 0.001$) (Table 3).

4 | DISCUSSION

Salivary IL-17, OPG, RANKL, and RANKL/OPG showed high sensitivity and specificity to differentiate periodontal health from gingivitis and periodontitis. Similar pattern was observed in discriminating stable and unstable periodontitis. However, salivary OPG and RANKL/OPG did not show enough sensitivity to differentiate gingivitis from periodontitis. This case-control study was conducted to evaluate the potential use of the aforementioned salivary proteins as diagnostic biomarkers.

The latest classification of periodontal diseases and conditions defined the periodontium that restored healthy state after successful periodontal therapy as stable periodontitis.[1] This is differentiated from unstable cases either by detecting PPD > 4 mm or PPD equal to 4 mm exhibiting BOP.[1] The reliance on manual probing measuring periodontal parameter could result in errors such as applying excessive force that leads to bleeding or falsely recording deeper pocket depth. These limitations are common with probing technique due to several reasons.[27–30] Lacking the accuracy in differentiating stable and unstable periodontium could significantly alter the treatment plan from continuing supportive to active periodontal therapy or vice versa.[6] Therefore, precise diagnosis is imperative to correctly tailoring the treatment plan. Use of biomarkers available in oral fluids showed promising results over the last decades to differentiate periodontal health from disease.[31]

Saliva was the oral fluid of choice due to ease of collection without causing discomfort to the patient, can be collected in sufficient volume, and the presence of a wide range of biomarkers that accurately reflecting several local and systemic conditions.[32,33] However, salivary biomarkers are more useful for screening purposes

TABLE 2 Area under the curve (AUC), sensitivity, specificity, and cut-off values for all groups.

Groups		IL-17[a]	RANKL[a]	OPG[a]	RANKL/OPG
Ctrl versus gingivitis	AUC	0.875	0.932	0.976	0.993
	Sensitivity	0.863	0.904	0.960	1.000
	Specificity	0.733	0.785	0.866	0.857
	Cut-off value	321.4	77.80	290.5	0.202
	p Value*	<0.001	<0.001	<0.001	<0.001
Ctrl versus periodontitis	AUC	0.879	0.951	0.913	0.977
	Sensitivity	0.860	0.911	0.860	1.000
	Specificity	0.800	0.857	0.800	0.851
	Cut-off value	322.2	79.48	351.2	0.198
	p Value*	<0.001	<0.001	<0.001	<0.001
Gingivitis versus periodontitis	AUC	0.666	0.706	0.532	0.527
	Sensitivity	0.604	0.822	0.520	0.555
	Specificity	0.590	0.523	0.480	0.619
	Cut-off value	382.8	87.45	135.8	0.628
	p Value*	0.03	0.007	0.65	0.73
Stable versus unstable periodontitis	AUC	0.924	0.953	0.889	0.964
	Sensitivity	1.000	0.909	0.880	1.000
	Specificity	0.850	0.956	0.840	0.782
	Cut-off value	378.7	110.2	149.6	0.433
	p Value*	<0.001	<0.001	<0.001	<0.001

Abbreviations: Ctrl, control (healthy periodontium); IL-17, interleukin-17; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor-κB ligand.
[a]Concentration in pg/mL.
*Significant difference at $p < 0.05$.

TABLE 3 Correlations between salivary biomarkers.

	IL-17	RANKL	OPG
IL-17			
r	-	0.545	−0.364
p Value*	-	<0.001	0.02
RANKL			
r	0.545	-	−0.496
p Value*	<0.001	-	0.001
OPG			
r	−0.364	−0.496	-
p Value*	0.02	0.001	-

Abbreviations: IL-17, interleukin-17; OPG, osteoprotegerin; *r*, correlation coefficient; RANKL, receptor activator of nuclear factor-κB ligand.
*Significant difference at $p < 0.05$ by Pearson's correlation assay.

rather than providing site-specific information which are more suitable for monitoring disease activity and prognosis.[34,35]

Bone resorption is a hallmark of periodontitis which is enhanced by upregulation of RANKL and concomitant downregulation of OPG expression; thereby, activating osteoclasts. Recent studies highlighted IL-17 as one of cytokines involved in bone-destruction process by increasing RANKL expression and decreasing OPG level.[36] Findings from this study supported this relation in which IL-17 was positively correlated with RANKL but negatively associated with OPG. Current study showed significant upregulation in the concentration of salivary IL-17 in gingivitis and periodontitis groups in comparison to Ctrl. This is in consistency with several studies that reported significant increase in the level of salivary IL-17 in participants with periodontal disease as compared to Ctrl.[11,37,38] In addition, circulating inflammatory cells in subjects with periodontitis showed deficiency that increased their tendency to abnormally producing IL-17 in the inflamed gingival tissue.[39]

RANKL is one of the distinctive inducers of osteoclastic activity which is highly increased in autoimmune and inflammatory diseases associated with bone resorption such as periodontitis. The level of RANKL is significantly upregulated in periodontitis in oral fluids including gingival crevicular fluid (GCF) and saliva than subjects with healthy periodontal state.[18,40] Indeed, the upregulation of RANKL is not the only requirement needed to initiate bone-destructive events of periodontitis but the downregulation of another regulatory protein, OPG, is crucially required. A previous clinical study nominated

salivary OPG as one of the potential biomarkers that can be used for monitoring periodontal disease.[41] Available literature indicates that the level of salivary OPG is significantly differ in health than disease; however, one study reported the opposite.[42] This could be attributed to the case definition of healthy controls who exhibited sites with loss of attachment and increasing PPD.

Belibasakis and Bostanci suggested that RANKL/OPG ratio can be effectively used as a biomarker for diagnosing periodontitis. In addition, this ratio can also indicate active bone resorption at molecular level following periodontal treatment.[43] Similarly, high diagnostic potential of RANKL/OPG system was observed in this study. However, this ratio did not differentiate between gingivitis and periodontitis while salivary RANKL alone was able to discriminate between them. This could be due to overlapping concentration of OPG in salivary samples of periodontitis and gingivitis in this study could affected the accuracy of RANKL/OPG ratio.

Increasing sample size, monitoring the level of the selected salivary biomarkers following periodontal therapy, and measuring their levels in GCF are recommended to overcome the limitations of this study. Introducing IL-17 as a biomarker for diagnosis of periodontal disease received limited attention by the previous studies which was investigated by the current study. Although the potential use of the salivary biomarkers as diagnostic tools was promising, further clinical studies are required to support the recent findings.

5 | CONCLUSION

Salivary IL-17, RANKL, OPG, and RANKL/OPG system are potential candidates for differentiating periodontal health and disease. In addition, these biomarkers exhibited the ability to discriminate pathologically reduced but stable periodontium from unstable periodontitis.

AUTHOR CONTRIBUTIONS

Marwa A. Abdullameer: data curation; investigation; methodology; validation; writing – original draft. **Ali A. Abdulkareem**: conceptualization; formal analysis; project administration; supervision; visualization; writing – review & editing.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The research followed the tenets of the Declaration of Helsinki. The Ethics Committee of college of Dentistry, University of Baghdad approved this study (Ref. 532, 17/04/2022, Project # 532622). All participants entered the study after they were fully informed of the process and signed written consent.

TRANSPARENCY STATEMENT

The lead author Marwa A. Abdullameer affirms that this manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned (and, if relevant, registered) have been explained.

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