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Potential of Salivary IL-36γ, IL-38, and RANKL in Differentiating Periodontal Health from Periodontitis

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Abstract

Objective: To evaluate the diagnostic potential of combined salivary biomarkers RANKL, IL-36γ, and IL-38 in distinguishing individuals with generalized unstable periodontitis from those with a healthy periodontium, by analyzing their concentrations and correlation with clinical periodontal parameters**. Material and Methods**: A total of 120 participants were included in this case-control study, comprising 90 subjects with generalized unstable periodontitis and 30 periodontally healthy controls. Clinical periodontal measurements, PI, PPD, CAL, and BOP, were recorded. Before data collection, inter- and intra-examiner calibration were performed on five subjects each, using kappa and intraclass correlation coefficients to ensure measurement reliability (>70% for BOP and >90% for PPD and CAL). Unstimulated saliva samples were collected, preserved in antiprotease solution, and stored on ice, then frozen for laboratory analysis. Salivary concentrations of RANKL, IL-36γ, and IL-38 were measured using ELISA kits. Data analysis included comparison between groups, correlation with clinical parameters, and diagnostic evaluation through sensitivity, specificity, and AUC analysis. Data were analyzed using SPSS v29 and GraphPad Prism v9. Normality was assessed using the Shapiro-Wilk test. Parametric tests (ANOVA, t-test) and non-parametric tests (Kruskal-Wallis, Mann-Whitney) were applied as appropriate. Correlations were evaluated using Spearman's test. Diagnostic accuracy was assessed through ROC curve analysis and AUC values, with statistical significance set at $p < 0.05$. **Results**: RANKL and IL-36γ salivary levels in periodontitis were significantly increased compared to healthy controls, while IL-38 levels were significantly reduced ($p < 0.001$). RANKL peaked in stage III, while IL-36γ was highest in stage IV. Conversely, IL-38 was consistently lower in both disease stages. Only IL-36γ with CAL correlation was a significant positive, while other clinical correlations were not statistically significant different. ROC analysis demonstrated excellent diagnostic accuracy for all three biomarkers in differentiating periodontal health from disease, with AUC values of 0.944 (RANKL), 0.982 (IL-36γ), and 0.960 (IL-38), along with high sensitivity and specificity. **Conclusion**: Salivary levels of RANKL, IL-36γ, and IL-38 demonstrated strong potential as non-invasive biomarkers for distinguishing generalized unstable periodontitis from periodontal health. Their diagnostic accuracy supports their utility in early detection and monitoring, although they showed limited ability to differentiate between periodontitis severity stages.

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Introduction

Periodontitis is one of the most common worldwide oral diseases [1]. Between 2011 and 2020, periodontitis in dentate adults was estimated to be around 62%, and severe periodontitis 23.6%. These results show an unusually high prevalence of periodontitis compared to the previous estimates from 1990 to 2010 [2].

Periodontal disease is a chronic, multifactorial inflammatory condition that affects the supporting structures of the teeth. It is primarily triggered by microbial plaque biofilm and modified by host immune-inflammatory responses, environmental, and genetic factors [3]. If left untreated, it may lead to progressive tissue destruction, alveolar bone loss, and ultimately, tooth loss [4].

Generalized unstable periodontitis represents an advanced stage of periodontal disease characterized by rapid progression and tissue breakdown. Early detection and accurate diagnosis are critical to managing

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this condition effectively and preventing irreversible damage [5]. Conventional periodontal diagnostics are mainly based on clinical parameters such as probing pocket depth (PPD), clinical attachment level (CAL), bleeding on probing (BOP), and plaque index (PI). While these indicators provide useful information, they often reflect past tissue damage and do not accurately capture current disease activity or risk of progression [6].

Recent advances in molecular diagnostics and salivary proteomics have opened new possibilities for non-invasive and real-time periodontal disease assessment. Saliva is increasingly recognized as a diagnostic fluid due to its accessibility, non-invasive collection method, and the presence of biomarkers that reflect both systemic and oral health conditions [7,8].

Among these biomarkers, Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL) has been identified as a key molecule in bone metabolism and periodontal destruction [9]. Interleukin-36γ (IL-36γ) is a pro-inflammatory cytokine involved in mucosal immunity and inflammation [10], while Interleukin-38 (IL-38) plays an anti-inflammatory role by regulating immune responses and potentially protecting tissues from excessive damage [11].

Therefore, the simultaneous evaluation of these salivary biomarkers may offer a more accurate and early diagnostic approach for periodontal disease compared to traditional methods. We aim to analyze the diagnostic potential of salivary RANKL, IL-36γ, and IL-38 in differentiating healthy periodontium from generalized unstable periodontitis. It further seeks to assess the correlation with the clinical periodontal parameters, as well as determine their diagnostic accuracy.

Material and Methods

Study settings

The multicenter study, a case-control observational study, was conducted at the Periodontics Departments of the College of Dentistry, University of Kirkuk, the Kirkuk Specialized Dental Center, and AL-Haweeja Health Center, Kirkuk, Iraq, from November 2023 to October 2024.

A total of 120 participants were recruited for the study, comprising 61 females (50.83%) and 59 males (49.17%), with a female-to-male ratio of approximately 1.03:1. The participants were categorized into two groups: healthy periodontium (n=30) and unstable generalized periodontitis (n=90) based on the latest periodontal disease and condition classification.

Sample Size Calculation

Based on previously reported mean and standard deviation (SD) values of salivary RANKL concentrations, one of the primary biomarkers assessed in this study, the sample size was calculated. Accordingly, the RANKL concentration in healthy individuals was estimated at 73.6 pg/mL, compared to 128.9 pg/mL in individuals with periodontitis. These values were used to assess the required sample size using ChatGPT-based statistical tools. The minimum calculated sample size for the periodontitis group was 80 subjects. To account for potential attrition, this number was increased to 90. Similarly, the control group was initially estimated to require 20 participants and was subsequently increased to 30 to minimize the risk of attrition bias.

Study Population

Consistent with the 2017 classification and the eligibility criteria of this study, subjects were grouped as:

1.

Healthy periodontium group: the diagnostic criteria for periodontal health on an intact periodontium included: BOP <10%, PPD ≤3 mm, and no evidence of alveolar bone loss clinically.

The unstable generalized periodontitis group was defined by one or more of the following criteria: sites with PPD ≥ 5mm or 4 mm with positive BOP, BOP >10%, and involving ≥30% of teeth. Interdental Clinical Attachment Loss (CAL) detectable at ≥2 non-adjacent teeth, or buccal or oral CAL ≥3 mm with PPD >3 mm, present at ≥2 teeth.

Eligibility Criteria

Participants were systemically healthy individuals (aged ≥18 years) diagnosed with either a healthy periodontium or unstable generalized periodontitis. All were, had at least 20 natural teeth (excluding third molars), and had not taken any medications in the three months before enrolment.

Exclusion criteria included systemic diseases (e.g. rheumatoid arthritis, cardiovascular disease, diabetes), smoking or alcohol use, pregnancy or lactation, presence of oral risk factors (e.g. carious or perio-endo lesions), use of orthodontic appliances, prior periodontal treatment within six months, or medication use (e.g. antibiotics, corticosteroids, biologics, or anti-inflammatories) within the last three months.

Ethical Approval

The research methodology employed in this study adhered to the Declaration of Helsinki and its subsequent amendments concerning human subject research, as well as the STARD 2015 (Standards for Reporting of Diagnostic Accuracy Studies). The study was approved by the Ethics Committee of the University of Baghdad's College of Dentistry (reference number: 892, Project number: 892624, Date: 11-1-2024).

Reliability Analysis

To ensure measurement reliability, calibration sessions were conducted before data collection. Inter- and intra-examiner calibration were performed on five subjects each, with a minimum of two hours between repeated assessments. PPD, BOP, and CAL were recorded at buccal/labial sites. Agreement for BOP was assessed using the kappa statistic (>70% inter-examiner, >75% intra-examiner), while PPD and CAL reliability were confirmed by intraclass correlation coefficients exceeding 90%.

Salivary Collection Procedure

Unstimulated whole saliva was collected before clinical examination using the passive drooling method [12]. Participants refrained from food, drink, and oral hygiene for at least two hours and rinsed their mouths with water before sampling [13]. Saliva was passively drooled into sterile tubes over 2–4 minutes [14].

Samples were coded, treated with a protease inhibitor, and stored on ice. They were centrifuged at 1000 rpm for 10 minutes, and 500 µL of the supernatant was transferred to labeled Eppendorf tubes and stored at −20°C until ELISA analysis [15].

Periodontal Examination

Periodontal status was determined according to the previously established criteria, after saliva collection. A calibrated examiner performed full-mouth periodontal charting (PI, BOP, PPD, and CAL) using a UNC-15 periodontal probe (Medesy, Italy).

The charting had begun at the distal surface of the upper right second/last molar and proceeded mesially, then lingually to cover all tooth surfaces. BOP, PPD, and CAL were assessed simultaneously. The probing force was standardized (20–25 g) as per calibration sessions (16). Consequently, periodontitis staging and grading followed the 2017 classification guidelines, based on the site with the highest CAL

Laboratory Analysis of Salivary Biomarkers

After data collection was completed, frozen saliva samples were transported to the laboratory, thawed at room temperature, and 100µm of the supernatant was collected for analyzing protein biomarkers. Salivary levels of IL-36γ, IL-38, and RANKL were measured using the commercially available ELISA kits.

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IL-36γ: Cloud-Clone Corp. ELISA kit, product no. SEL621Hu (Cloud-Clone Corp., Wuhan, China).
-Sensitivity: <6.5pg/mL and detection range 15.6-1000 pg/mL.
IL-38: Cloud-Clone Corp., Wuhan, China ELISA Kit, Product No. SEQ458Hu (Cloud-Clone Corp., Wuhan, China).
-Sensitivity: 46.88pg/mL and the detection Range: 78.13-5000pg/mL.
RANKL: Human RANKL ELISA Kit from Elabscience. Their Product No is E-EL-H5813 (headquartered in Houston, USA).
-Sensitivity: 9.38 pg/mL, and the detection range is 15.63-1000 pg/mL.
Each 96-well plate was pre-coated with a monoclonal antibody specific to the target analyte, employing the sandwich ELISA technique. All procedures followed the manufacturer's protocol.

Statistical Analysis
For continuous variables, central tendency and dispersion were quantified using mean values and standard deviation (SD). The Shapiro-Wilks test was applied to evaluate the normality of data distribution. Intergroup comparisons were performed using an independent sample t-test, Mann-Whitney U test, and chi-square test. Within each group, Spearman correlation analysis was conducted to examine relationships between variables. Positive and negative predictive values were determined through contingency table analysis. Statistical significance was defined as $p<0.05$.
The sensitivity, specificity, and optimal cut-off points for individual biomarkers, as well as their ratios, were evaluated using receiver operating characteristic (ROC) curve analysis and the area under the curve (AUC). For this purpose, the concentrations of each biomarker were dichotomized, assigning "0" to the healthy group and "1" to the periodontitis group. All statistical analyses were performed using GraphPad Prism software (version 9.0). A p-value of less than 0.05 was considered statistically significant.

Results

Out of the initially assessed 600 individuals, 120 participants met the inclusion criteria and were divided into two groups: 90 with generalized unstable periodontitis and 30 periodontally healthy controls. The demographic analysis revealed significant differences in age and gender between the groups ($p < 0.001$), with the periodontitis group having a higher mean age and a greater proportion of males (Table 1).

Clinical Parameters
All clinical periodontal parameters (PI, BOP, PPD, and CAL) were significantly higher in the periodontitis group compared to the control group ($p < 0.001$). Stage III and IV periodontitis showed progressively increased severity, with stage IV presenting the highest mean values in BOP and CAL (Figure 2).

Salivary Biomarker Levels
RANKL levels were significantly higher in the periodontitis group (261.61 ± 96.27 pg/ml) than in controls (125.62 ± 53.92 pg/ml), with the highest levels observed in stage III (Table 2). Similarly, IL-36γ levels were also significantly elevated in periodontitis (340.77 ± 90.61 pg/ml) compared to controls (138.18 ± 50.68 pg/ml), while peaking in stage IV (Table 3). Conversely, IL-38 levels, in contrast, were significantly lower in the periodontitis group (63.52 ± 21.24 pg/ml) than in controls (142.80 ± 28.53 pg/ml) (Table 4).

Correlations of Salivary Biomarkers with the Periodontal Parameters
IL-36γ showed a significant positive correlation with CAL ($r = 0.095$, $p = 0.024$), whereas statistically non-significant correlations were found between RANKL or IL-38 and clinical parameters (Table 5). Additionally, within stage III, IL-36γ showed significant negative correlations with PI and BOP. In stage IV, BOP was significantly and positively correlated with RANKL (Table 6).

Inter-Biomarker Correlations
A strong positive correlation was observed between RANKL and IL-36γ in both control ($r = 0.671$) and periodontitis groups ($r = 0.560$, $p < 0.001$). On the other hand, IL-38 was negatively correlated with both of them in the control group, while a significant positive correlation was observed in the periodontitis group (Table 7).

Diagnostic Performance
ROC analysis demonstrated the excellent diagnostic performance of the three biomarkers in distinguishing between periodontal health and unstable generalized periodontitis. There were the AUCs: RANKL (0.944), IL-36γ (0.982), and IL-38 (0.960). On the flap side, sensitivity ranged from 94.59% to 98.88%, and specificity ranged from 93.10% to 96.55%. However, the three biomarkers failed to significantly distinguish between stage III and IV (AUC < 0.60).

Discussion
The present study evaluated the diagnostic potential of salivary IL-36γ, IL-38, and RANKL in distinguishing periodontal health from generalized unstable periodontitis using ROC curves, sensitivity, and specificity concerning clinical periodontal parameters. The findings revealed high sensitivity and specificity for these biomarkers in differentiating health from disease, although their ability to distinguish between different periodontitis stages remains limited.
Despite advancements in oral health care, periodontitis remains highly prevalent [17]. The Global Burden of Disease 2021 Study reported over 1 billion cases worldwide, with severe forms expected to rise by over 44% by 2050 [18]. This persistent burden is attributed to a reliance on restorative rather than preventive care, underscoring the urgent need for point-of-care diagnostics and personalized prevention strategies [19].
Periodontitis typically begins as gingivitis caused by biofilm accumulation, leading to dysbiosis, a shift toward pathogenic bacteria, and triggering a host immune-inflammatory response [20,21]. Current diagnostic methods, including probing and radiographs, reflect past damage and lack sensitivity to ongoing inflammation or microbial changes [22]. Their subjectivity and variability highlight the need for more objective tools [23], prompting the inclusion of novel diagnostic domains in recent periodontal classifications [22].
A forward-looking approach aims to detect periodontitis before clinical signs emerge, utilizing biomarkers as predictive and prognostic indicators. They can identify disease presence, progression, or treatment response [24]. Salivary biomarkers like interleukins and RANKL have shown promise as non-invasive diagnostic tools. RANKL demonstrates excellent sensitivity and high specificity for distinguishing healthy from diseased states [25,26]. Similarly, IL-36γ, part of the IL-1 cytokine family, plays a key role in periodontal inflammation and bone loss, making it a significant emerging marker in chronic periodontal disease [27,28].
On the other hand, IL-38 can act as an inhibitor of IL-36 pro-cytokines (IL-36α, IL-36β, and IL-36γ), as it partially blocks IL-36R, which is activated via these pro-cytokines and inhibited by IL-36Ra [29-31]. Therefore, the IL-38/IL-36R axis suggests the potential therapeutic benefit of IL-38 in inflammatory autoimmune diseases, primarily through its anti-inflammatory role in the development and resolution of these diseases [32].
Regarding periodontal disease, contrasting findings on IL-38 levels in different studies highlight its complex role in periodontal inflammation. While increased GCF and saliva levels in one study suggest its potential as a marker for active disease [33], the decreased salivary levels in another study indicate that

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further research is needed to fully understand its diagnostic utility [34].

Recently, studies demonstrated that individuals with periodontitis exhibited significantly lower salivary IL-38 levels compared to healthy individuals, suggesting a potential inverse relationship between IL-38 levels and the severity of periodontal disease [33,34]. Conversely, in another study, interleukin-36γ and IL-38 have been shown to have elevated salivary levels in periodontitis compared to healthy periodontium or gingivitis. Their salivary levels correlate with clinical periodontal parameters, suggesting a potential role in diagnosing periodontitis and assessing disease activity [33].

On the flap side, saliva contains a variety of biomarkers, including proteins, DNA, and RNA, which can be used to diagnose multiple diseases, such as cancer, cardiovascular diseases, and oral diseases [35,36]. Along with this, due to its elements that reflect the activity of all periodontal sites, saliva content reflects a consensus 'whole mouth' inflammatory status rather than at active disease sites, as with GCF analysis [37]. Besides, saliva collection, especially in children and large populations, is a non-invasive, easy-to-handle, and suitable method for mass screening [35,38].

Moreover, quantifying cytokines is particularly crucial for establishing threshold levels of the selected biomarkers, which are essential for developing a chairside clinical tool. Therefore, the ELISA technique was chosen for quantifying biomarker concentrations due to its user-friendliness, high sensitivity, and specificity, which are attributed to the unique interaction between the antibody and antigen [39-41]. Alongside its excellent reproducibility and quantitative detection ability for pro-inflammatory and anti-inflammatory cytokines [37,39,41,42]. Not only that, but ELISA can also be adapted for various applications, including the detection of cancer biomarkers and cytokines, and is compatible with different sample types such as blood, serum, and plasma [42-44].

Additionally, ELISA, by detecting specific biomarkers, is extensively used for early disease diagnosis, including Alzheimer's and cancer [39,43,45]. Therefore, the technique employed in both clinical diagnostics and research laboratories is for its reliability and adaptability [44,46].

Demographic Distribution and Group Characteristics

A sample size of 120 participants was calculated, comprising a control group with healthy periodontium (n = 30) and a case group with generalized unstable periodontitis (n = 90). The unequal distribution of participants is attributed to several factors related to study design and participants' characteristics.

Many studies use specific matching criteria to form control groups, such as age, sex, and socioeconomic status, which can limit the number of eligible control participants. For instance, in a study on periodontitis and rheumatoid arthritis, controls were matched 1:1 with periodontitis participants based on these criteria, which can inherently lead to unequal distribution if the pool of eligible controls is smaller [47].

Similarly, in our research, the control group criteria involved systemically and periodontally healthy people, aged >18 years, who had a BOP <10%, with more than 20 teeth in their mouth, and were free from current or previous periodontitis. Ordinarily, these criteria resulted in a lower percentage of people in the health center's community compared with the periodontitis group criteria.

Furthermore, the prevalence of periodontitis in some populations can lead to a higher number of participants with the condition compared to those without. Likewise, by using the 2017 classification, periodontitis was found in 36.5% of the sampled Iraqi population, with severe cases (stages III and IV) being the most common, accounting for 77.3% of periodontitis cases [48].

On the other hand, there were significant demographic differences between the groups, including gender and age (Table 1). The control group consisted predominantly of females (72.4%) and younger individuals (26.93 ± 4.14 years), while the periodontitis group was older (42.79 ± 12.39 years) with a higher percentage of males (56.2%). These differences are consistent with the well-established understanding that periodontitis is more prevalent in older individuals [49-52], and may be more pronounced in males [50,53-55]. Alongside other factors such as smoking and low socioeconomic status, male was identified as a significant risk factor for periodontitis [51,53]. Although the smokers were excluded from the current study, the socioeconomic status was not, and the gender selection was random.

What is more, the distribution of periodontitis stages (Table 2) in the periodontitis group showed that the majority of participants were in advanced stages (Stage III, 39%, and Stage IV, 31.4%), which is significant in the context of understanding the severity of the condition in this cohort. The relatively low proportion of patients in Stage I (2.1%) and Stage II (4.2%) may suggest a study bias towards individuals with more severe forms of periodontitis, potentially due to the inclusion criteria favoring those with more noticeable clinical symptoms, such as involving only periodontitis patients with the generalized extent and unstable status.

Otherwise, this distribution is consistent with the various studies, which indicate that a significant portion of individuals are in advanced stages of the disease. In a Norwegian population, Stage III and IV periodontitis were observed in 17.6% of the study population, with severe forms primarily occurring after 60 years of age [56]. Similarly, in a rural Chinese population, more than half of the individuals had Stage III/IV periodontitis, with a rapid progression noted [57]. Likewise, in a Sámis population in Northern Norway, 20.1% were in Stage III/IV [58]. Besides, the prevalence of severe periodontitis increases with age [57]. As well as, individuals with Stage III/IV periodontitis reported more impairment in quality of life and masticatory function [59,60], which led to a higher percentage of those patients in healthcare centers.

In the context of comparisons between the study groups, clinical periodontal parameters (PL, BOP, PPD, and CAL) showed significant differences between the healthy control and periodontitis groups. Both PLI and BOP were significantly higher in the periodontitis group, especially in stages III and IV, compared to the control group (Figure 2, A, B). These findings support the expected link between clinical inflammation and the severity of periodontitis, as higher PLI and BOP scores are associated with increased severity. These parameters reflect the inflammatory condition of the gingival tissue and are used to evaluate the progression of periodontal disease [61-63].

The PPD and CAL also showed significant differences between stages III and IV (Figure 2, C, D), highlighting the increased severity of periodontal destruction in more advanced stages of the disease. Various factors, including host responses, influence this progression [64], oxidative stress [65,66], microbial factors [67], functional impairments [60], and systemic conditions [68,69]. Studies indicate that higher CAL values correlate with more severe periodontal disease [61,62,70,71]. Likely, increased PPD is consistently associated with higher disease severity [61-63,72].

Salivary Biomarkers in Periodontal Health and Disease

Salivary biomarkers, specifically RANKL, IL-36γ, and IL-38, were examined to assess their potential diagnostic biomarkers, differentiating between periodontal health and generalized unstable periodontitis.

RANKL

In the context of periodontitis, literature has demonstrated that RANKL is a key mediator in bone resorption and a hallmark of periodontal disease [73,74], primarily through its

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expression by activated T and B lymphocytes [75,76], osteocytes, and macrophages [77], and its regulation by immune responses and inflammatory cytokines [75]. Furthermore, the increased RANKL expression is attributed to the periodontopathic bacteria, such as Porphyromonas gingivalis, which are known to exacerbate periodontal disease [78].

In our study, RANKL demonstrated an AUC of 0.941, with a sensitivity of 97.22% and specificity of 93.10%, suggesting a highly effective capacity of RANKL in differentiating healthy periodontium from generalized unstable periodontitis (Table 9) (Figure 3-A). Consistent with the previous study, RANKL exhibits perfect sensitivity (1.00) and high specificity (0.92) in determining these conditions, making it a reliable biomarker for early detection and management of periodontal diseases [79].

Moreover, RANKL levels were significantly higher in the periodontitis group compared to the control group, with stage III showing a slightly higher mean level than stage IV (Table 3). This elevation is consistent with available literature, which demonstrated that RANKL levels, both in saliva and gingival tissues, are significantly higher in patients with periodontitis compared to healthy controls has been observed in various forms of periodontitis [78,80-85], indicating its potential as a marker for periodontal disease [79,81,82].

Furthermore, in our study, RANKL levels correlate with PI, and PPD positively but non-significantly (Table 6). This is consistent with various studies, where RANKL levels are elevated in periodontal disease, characterized by the correlation between RANKL levels and these clinical parameters is often not statistically significant, indicating variability in individual responses or other influencing factors [73,81,86].

Treatments such as antimicrobial photodynamic therapy and the use of resveratrol-containing mouthwash have been shown to reduce RANKL levels and improve periodontal parameters. However, the changes in RANKL levels do not always correlate directly with changes in PI and PPD [87,88]. Despite that, these factors were considered excluded criteria in this study. Therefore, we suggested that while RANKL is an important factor in periodontal disease, its levels alone may not be a reliable predictor of clinical severity as measured by PI and PPD. Other factors, including individual variability and additional inflammatory mediators, likely play significant roles.

In addition, the relationship between clinical parameters such as BOP and CAL with RANKL levels has been explored (Table 6), indicating a negative, non-significant correlation between BOP and CAL with RANKL levels. Likewise, various studies indicate a negative correlation between BOP and CAL with RANKL levels, which is also not statistically significant. These suggest that changes in BOP and CAL do not strongly predict changes in RANKL levels in the context of periodontitis [86,88,89].

Alternatively, other studies demonstrate that the higher RANKL levels correlate positively with clinical indicators of periodontal diseases, such as (PPD) and (CAL), suggesting that RANKL could act as a diagnostic marker for periodontal disease [78,81,84,85]. As well, detecting RANKL in saliva makes it a non-invasive biomarker for diagnosing periodontal disease, with high sensitivity and specificity in distinguishing between periodontal health and disease [79,81].

Additionally, in comparison with stages III and IV of periodontitis (Table 3), RANKL levels are significantly higher than in healthy controls, with stage III showing slightly higher levels than stage IV. Consistent with previous literature, it suggests a nuanced role of RANKL in different stages of periodontitis [26]. In other words, suggests that RANKL could be used as a biomarker for the severity of periodontitis, differentiating between different stages. Although in this study, RANKL lacks discrimination between stage III periodontitis and stage IV periodontitis (Table 9) (Figure 3), it could be because both stages represent severe forms of the disease, or a larger sample size is needed for that purpose.

IL-36γ

As RANKL, the current study showed IL-36γ with an even higher AUC of 0.979, with identical sensitivity (97.22%) and slightly higher specificity (96.55%), reinforcing its robust role as a diagnostic marker for generalized unstable periodontitis (Table 9) (Figure 3). In another study, a combination of IL-1β with IL-36γ is particularly effective in distinguishing periodontitis from periodontal health, suggesting its utility as part of a biomarker panel for early detection and monitoring of periodontal disease. Despite its high sensitivity in detecting periodontitis, IL-36γ exhibits a relatively low specificity when identifying individuals without periodontitis [34].

Recently, studies have consistently shown that IL-36γ levels are significantly elevated in patients with periodontitis compared to healthy individuals, particularly pronounced in more advanced stages of the periodontitis, where there is a greater degree of tissue destruction and bone loss, as stages III and IV [27,33,90]. Our finding that IL-36γ levels followed a similar pattern as RANKL, with elevated levels in the periodontitis group, particularly in stages III and IV (Table 4), reflects its role in the inflammatory response during periodontitis, consistent with the previous studies, given its involvement in pro-inflammatory signaling pathways.

As an inflammatory response indicator, IL-36γ enhances the expression of other pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α). While it is a bone loss biomarker associated with periodontitis, it increases the RANKL/OPG ratio [27,91]. Additionally, it promotes neutrophil chemotaxis, a crucial component of the immune response at the oral barrier, which is mediated through fibroblasts and is a key factor in the progression of periodontitis [91].

Moreover, IL-36γ enhances the MAPK and TLR4 signaling pathways associated with chronic periodontitis; so that IL-36γ not only indicates disease presence but actively participates in its progression [91,92]. Therefore, targeting IL-36γ and its pathways could offer new therapeutic avenues for managing periodontitis. Hence, inhibiting IL-36γ activity decreased neutrophil infiltration and bone resorption in experimental models, suggesting its potential as a therapeutic target [90,91].

In harmonious, when examining the correlation between clinical periodontal parameters and salivary biomarkers (Table 6, Table 7), it was observed that CAL was positively correlated with IL-36γ in the periodontitis group, which suggests a link between the inflammatory response (as indicated by IL-36γ levels) and clinical attachment loss, a key marker of periodontal destruction. Previously, a study has indicated a positive correlation between salivary IL-36γ levels and clinical periodontal parameters, such as PPD and CAL. This implies that periodontal disease severity increases IL-36γ levels, reflecting the inflammatory state of the periodontium [33].

Other clinical parameters, such as PLI, BOP, and PPD, did not show significant correlations with the biomarkers. This may indicate that these markers are more directly associated with the chronicity and severity of periodontal destruction rather than with early inflammatory responses.

Studies have identified a positive correlation between the concentrations of IL-36γ in oral fluids and certain clinical periodontal parameters, including probing depth and clinical attachment loss. This evidence highlights the potential of these biomarkers in assessing the activity and severity of periodontitis [33]. Conversely, in our study, BOP, the unique predictive test used by periodontists for routinely assessing the stability or progression of periodontitis [93], showed negative and non-significant correlations with IL-

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36γ salivary biomarkers (Table 6). In addition, IL-36γ is significantly elevated in periodontitis patients, correlated with the RANKL/OPG ratio, suggesting IL-36γ's involvement in perpetuating gingival inflammation and alveolar bone resorption, making it a potential therapeutic [27].

IL-38

In this study, IL-38 showed an AUC of 0.973, with perfect sensitivity (100%) and a specificity of 93.10% (Table 9). These findings suggest that IL-38 may have a superior ability to detect generalized unstable periodontitis, further supporting its role as a diagnostic biomarker, consistent with S. Kc et al. [94]. To our knowledge, this is the first study to evaluate the diagnostic potential of IL-38.

The contrasting findings on IL-38 levels in different studies highlight its complex role in periodontal inflammation. The increased levels of GCF and saliva in one study and of GCF in another one suggested its potential as a marker for active disease [33,95]. While the decreased salivary levels in another study [34] align with our findings, IL-38 was significantly lower in the periodontitis group compared to the healthy controls, and its levels were lower in stages III and IV (Table 5). This finding suggests that IL-38 might play an inhibitory role in periodontal inflammation, as its reduced expression could be a response to chronic inflammation in periodontal tissues.

The general trend supports the use of multiple biomarkers for better diagnostic precision. Combining multiple biomarkers, such as IL-1β, IL-6, and MMP-8, improved diagnostic accuracy for periodontitis. While RANKL, IL-36γ, and IL-38 are not specifically highlighted in these combinations, the general trend supports the use of multiple biomarkers for better diagnostic precision [94,96,97].

Aligning with previous studies, their gingival crevicular fluid, saliva, and serum levels correlate with disease severity and activity, providing a non-invasive means to assess periodontal health [26,33]. These findings support the potential of RANKL, IL-36γ, and IL-38 as biomarkers for the early detection and monitoring of periodontal disease. The sensitivity and specificity values reflect that these biomarkers can accurately identify patients with periodontitis and potentially assist in differentiating it from periodontal health across various disease stages. Therefore, combining IL-36γ, IL-38, and RANKL could serve as a comprehensive diagnostic biomarker panel for periodontal disease.

The determination of diagnostic thresholds for biomarkers is crucial for distinguishing between periodontal health and various stages of periodontitis [94,96,98]. Recent research highlights several promising biomarkers and their potential diagnostic applications. In clinical diagnostics [98,99], determining effective cut-off points for biomarkers is crucial, as they help patient stratification based on disease risk or treatment response [100,101].

The method selection for determining cut-off points should align with the biomarker's intended use, whether for diagnosis, prognosis, or screening. Different applications may require different statistical approaches [102]. While ROC curve analysis is widely used, it is a common method for determining cut-off points, particularly for diagnostic purposes. It involves selecting a point that maximizes sensitivity and specificity, often using the Youden index [103,104]. However, ROC curves are not ideal for prognostic biomarkers as they do not account for time-to-event data [102].

The proposed cut-off concentrations for differentiating periodontal health from periodontitis are 143.8 pg/ml, 196.9 pg/ml, and 67.05 pg/ml of RANKL, IL-36γ, and IL-38, respectively (Table 9) (Figure 3-A).

Additionally, proposed cut-off points for the biomarkers provide valuable insights into their diagnostic thresholds for identifying periodontal health and different stages of periodontitis. The cut-off points for the biomarkers RANKL, IL-36γ, and IL-38 were 143.8 pg/ml, 196.9 pg/ml, and 66.69 pg/ml, respectively (Table 9) (Figure 3-B), suggesting the potential to discriminate between periodontal health and periodontitis stage III. On the other hand, the cut-off points for these biomarkers were 161.3 pg/ml, 256.8 pg/ml, and 111.9 pg/ml, respectively. Suggesting a potential to discriminate between periodontal health and periodontitis stage IV, as illustrated in (Table 9) (Figure 3-C).

These cut-off concentrations further solidify the role of these biomarkers as effective diagnostic tools for identifying early and moderate stages of periodontitis. However, these biomarkers may need to be supplemented with other diagnostic approaches, such as clinical examination or advanced imaging techniques, to improve accuracy in distinguishing the most severe stages of periodontitis [105,106].

Despite their potential strong diagnostic performance in distinguishing between healthy periodontium and periodontitis, the biomarkers demonstrated much lower diagnostic accuracy when comparing stages III and IV of periodontitis. The AUC values between stages III and IV for all three biomarkers were low, with values 0.532 pg/ml, 0.580 pg/ml, and 0.540 pg/ml, for three biomarkers RANKL, IL-36γ, and IL-38, respectively (Table 9) (Figure 3-D) indicating that these biomarkers are ineffective in discriminating between these two advanced stages of periodontitis as AUC below 0.7 is typically regarded as poor or ineffective [106-108].

This lack of discrimination between stages of periodontitis may be because both represent severe forms of the disease, where the inflammatory and pathological processes may involve overlapping immunological pathways. This makes further distinctions more difficult using these biomarkers alone [109,110], or biomarker levels may reach saturation, reducing their effectiveness in distinguishing between different stages of periodontal disease [109,111].

Limitations and Future Directions

The findings of this study suggest the potential utility of salivary biomarkers in diagnosing periodontitis, although several limitations exist. The unequal distribution of participants between the control and periodontitis groups could introduce bias, particularly in the biomarker comparison across different periodontitis stages. Additionally, they were insufficient to distinguish between different severity stages of periodontitis (stage III vs. stage IV). Therefore, the relatively small sample size in the control group may limit the generalizability of these findings.

Furthermore, interpreting large datasets from salivary diagnostics can be complex, requiring advanced analytical techniques [35]. While many potential biomarkers have been identified, further research is needed to validate their effectiveness and reliability in clinical settings [96,100]. Future studies with larger, more balanced cohorts and longitudinal designs are recommended for investigating the role of these biomarkers in distinguishing between healthy and diseased states. Moreover, the integration of multiple salivary biomarkers may enhance early diagnosis and monitoring of periodontal disease in a non-invasive and patient-friendly manner. Therefore, salivary RANKL, IL-36γ, and IL-38 represent promising tools for future diagnostic protocols in personalized periodontal care.

Conclusion

Salivary RANKL and IL-36γ levels were elevated, while IL-38 was reduced in periodontitis, indicating their roles in periodontitis pathogenesis. ROC analysis revealed RANKL had the highest diagnostic accuracy. These findings support the potential use of RANKL, IL-36γ, and IL-38 as non-invasive biomarkers for early detection and monitoring of periodontal disease.

Conflict of Interest

No conflicts to declare.

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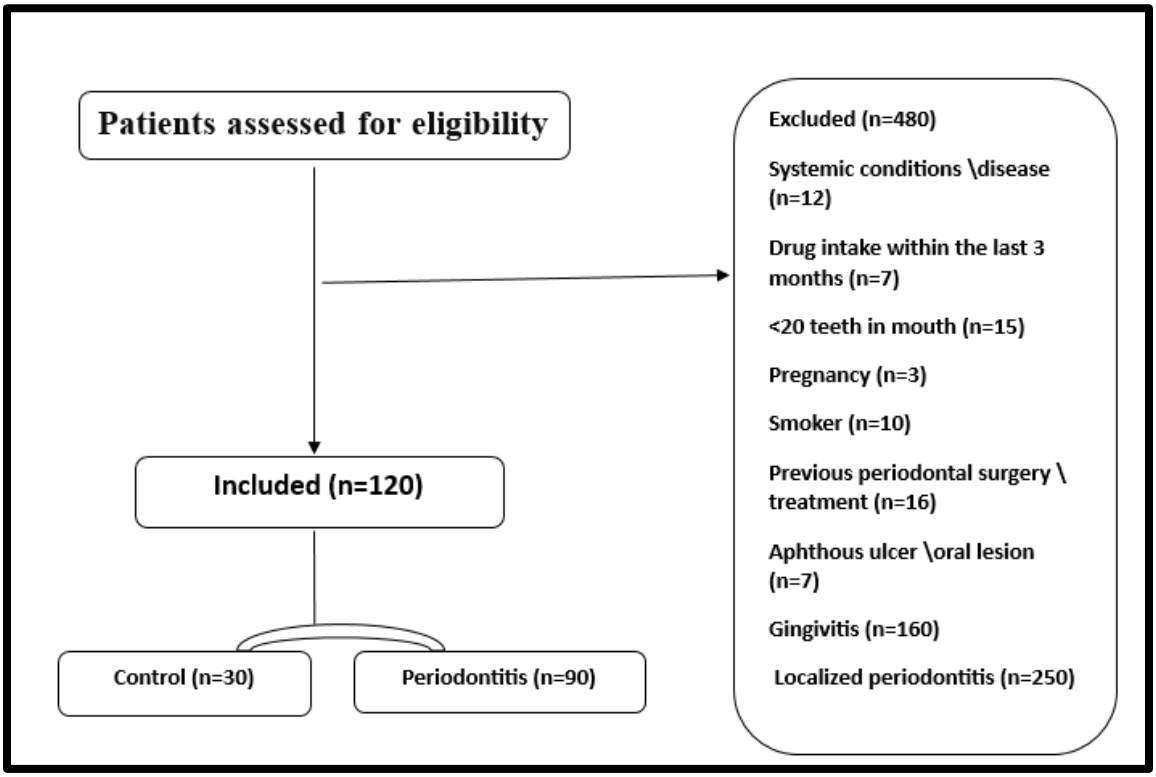


Figure 1. Flowchart of the study.

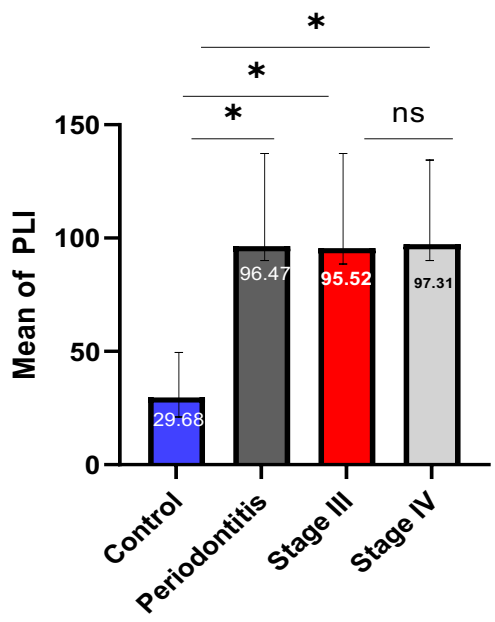
Table 1. Demographic variables of the study population.

	Total (N=120)	Control (n=30)	Periodontitis (90)	p-value
Sex				
Male N (%)	59(49.2%)	9(27.6%)	50(56.2%)	<0.001*
Female N (%)	61(50.8%)	21 (72.4)	40(43.8%)	
Mean ±SD		26.93± 4.14	42.79±12.39	<0.001**
Minimum		21	18	
Maximum		38	75	

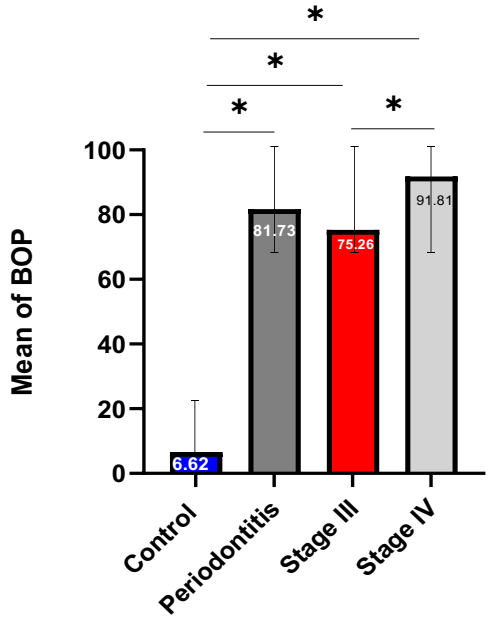
M: male, F: female; N: number; *Comparison done by chi-square; level of significance ≤ 0.05; Sig: significant. ** Independent t-test, Sig: significant; SD: standard deviation; Min: minimum; Max: maximum.

Table 2. Periodontitis stages distribution.

Periodontitis	Stage I	Stage II	Stage III	Stage IV
N	2	5	46	37
%	2.2%	5.5%	51.1%	41.1%



A



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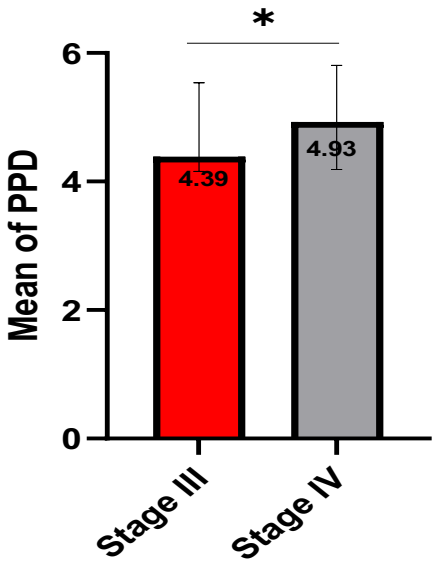

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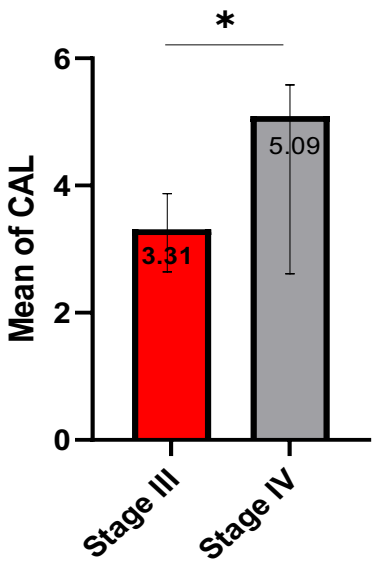

D

Figure 2. Comparisons of clinical periodontal parameters in periodontal health and disease. A) The PLI means between control Vs periodontitis and control Vs stage III, IV. B) The BOP means between control Vs periodontitis and control Vs stage III, IV. C) The PPD mean between stage III Vs IV. D) The CAL mean between stage III Vs IV.

Table 3. RANKL (pg\ml) among the study groups.

Biomarker	Groups	Mean	±SD	Min	Max	Comparison	p-value
RANKL (pg\ml)	**Control**	**125.62**	**53.92**	**87.54**	**353.08**		
	Periodontitis	261.61	96.27	126.95	914.55	C VS P*	<0.001
	Stage III	263.93	118.01	126.95	914.55	C VS PS**	<0.001
	Stage IV	256.91	72.03	160.64	506.13		

Abbreviations: C: control, P: periodontitis, PS: periodontitis stage, S-III, IV: stage III, IV
*comparison using Mann-Whitney test **; Comparison using Kruskal-Wallis test; level of significance ≤ 0.; SD: Standard deviation; Sig: Significant

Table 4. IL-36γ (pg\ml) among groups.

Biomarker	Groups	Mean	±SD	Min	Max	Comparison	p-value
IL-36γ (pg\ml)	**Control**	**138.18**	**50.68**	**33.11**	**318.11**		
	Periodontitis	340.77	90.61	169.56	753.86	C VS P *	<0.001
	Stage III	329.72	82.91	169.56	658.18	C VS PS**	<0.001
	Stage IV	359.53	98.61	254.34	753.86		

Abbreviations: C: control, P: periodontitis, PS: periodontitis stage, S-III, IV: stage III, IV
*comparison using Mann-Whitney test **; Comparison using Kruskal-Wallis test; level of significance ≤ 0.; SD: Standard deviation; Sig: Significant.

Table 5. IL-38 (pg\ml) among groups.

Biomarker	Groups	Mean	±SD	Max	Min	Comparison	p-value
IL-38 (pg\ml)	**Control**	**142.80**	**28.53**	**55.92**	**175.84**		
	Periodontitis	63.52	21.24	20.80	151.76	C VS P*	<0.001
	Stage III	61.63	20.42	20.80	129.89	C VS PS **	<0.001
	Stage IV	65.25	22.82	29.77	151.76		

Abbreviations: C: control, P: periodontitis, PS: periodontitis stage, S-III, IV: stage III, IV.
*comparison using Mann-Whitney test **; Comparison using Kruskal-Wallis test; level of significance ≤ 0.05; SD: Standard deviation; Sig: Significant

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Table 6. Correlation between clinical periodontal variables (%PI, % BOP, PPD, and CAL) with salivary biomarkers (RANKL, IL-36γ, and IL-38) levels in the unstable generalized periodontitis group.

Variables	RANKL		IL-36γ		IL-38	
	R	p-value	R	p-value	R	p-value
PI	0.078	0.468	-0.110	0.304	0.137	0.2
BOP	-0.023	0.828	-0.001	0.992	-0.121	0.257
PPD	0.052	0.631	0.178	0.24	0.036	0.739
CAL	-0.080	0.46	0.095	0.024	-0.100	0.299

Abbreviations: RANKL, Receptor activator of nuclear factor kappa-B ligand; IL-36γ, Interleukin 36γ; IL-38, Interleukin-38; BOP, bleeding on probing; PLI, plaque index; NS, non-significant; sig, significant; r, Spearman's correlation coefficient

Table 7. Correlation between clinical periodontal parameters with RANKL, IL-36, and IL-38 salivary levels in periodontitis stage III and stage IV.

Periodontitis severity	variables	RANKL		IL-36γ		IL-38	
		R	p-value	R	p-value	R	p-value
Stage III	PLI	0.199	0.191	-0.383	0.009	0.223	0.136
	BOP	0.054	0.723	-0.154	0.024	-0.137	0.362
	PPD	-0.005	0.975	-0.100	0.507	-0.089	0.558
	CAL	-0.167	0.273	0.201	0.18	-0.203	0.176
Stage IV	PLI	-0.067	0.697	0.273	0.103	0.127	0.452
	BOP	0.280	0.003	-0.131	0.441	-0.184	0.276
	PPD	0.216	0.2	0.171	0.31	0.174	0.304
	CAL	0.119	0.483	0.171	0.311	-0.014	0.933

Abbreviations: RANKL, Receptor activator of nuclear factor kappa-B ligand; IL-36γ, Interleukin-36γ; IL-38, Interleukin-38; PPD, probing pocket depth; CAL, clinical attachment level; NS, non-significant; sig, significant; r, Spearman's correlation coefficient

Table 8. Correlation between RANKL, IL-36γ, and IL-38 in control and periodontitis groups.

Interaction		RANKL		IL 38	
		R	p-value	R	p-value
Control	IL36γ	0.671	0.001	-0.476	0.012
Periodontitis	IL 36γ	0.560	0.001	0.632	0.001
Control	IL 38	-0.773	0.001	-	-
Periodontitis	IL38	0.482	0.001	-	-

Abbreviations: RANKL, Receptor activator of nuclear factor kappa-B ligand; IL-36γ, Interleukin 36γ; IL-38, Interleukin-38; sig, significant; r, Spearman's correlation coefficient.

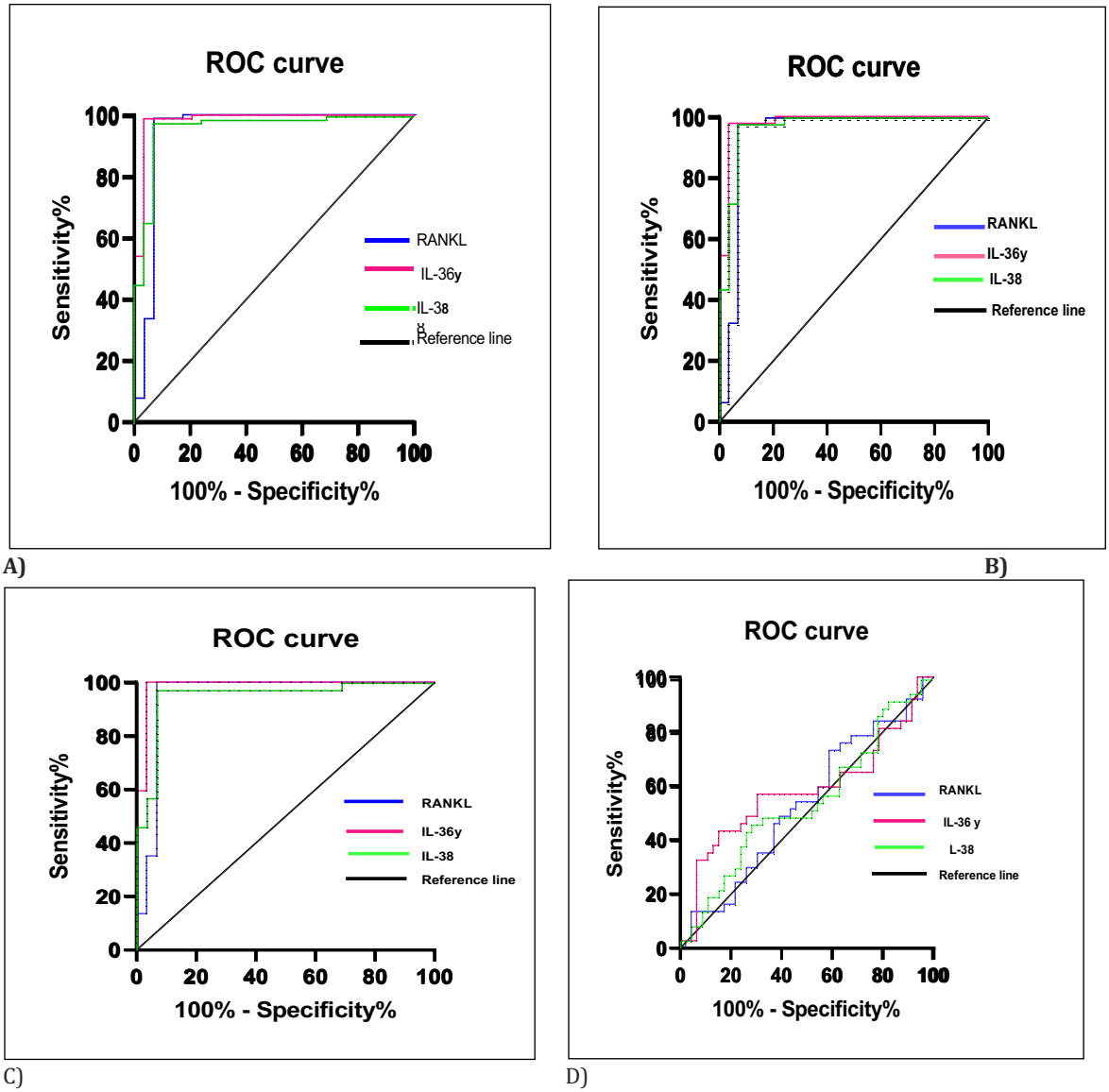

Figure 3. ROC curves of RANKL, IL-36, and IL-38, (A) Control Vs. Periodontitis (B) Control Vs. Stage III, (C) Control Vs. Stage IV (D) Stage III Vs. Stage IV.

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Table 9. Sensitivity, specificity, and cut-off points of RANKL, IL-36, and IL-38.

	Test Result Variable(s)	AUC		P-value	Optimal cutoff point	%Sensitivity	%Specificity
Control VS Periodontitis	RANKL	0.944	**Excellent**	<0.0001	143.8	98.88	93.10
	IL-36	0.982	**Excellent**	<0.0001	196.9	98.88	96.55
	IL-38	0.960	**Excellent**	<0.0001	67.05	65.17	96.55
Control VS Stage III	RANKL	0.942	**Excellent**	<0.0001	143.8	97.85	93.10
	IL-36	0.980	**Excellent**	<0.0001	196.9	97.83	96.55
	IL-38	0.967	**Excellent**	<0.0001	66.69	71.74	96.55
Control VS Stage IV	RANKL	0.947	**Excellent**	<0.0001	161.3	94.59	93.10
	IL-36	0.986	**Excellent**	<0.0001	256.8	97.30	96.55
	IL-38	0.949	**Excellent**	<0.0001	111.9	97.30	93.10
Stage III VS Stage IV	RANKL	0.532	**Fail**	0.614	238.5	54.05	52.17
	IL-36	0.580	**Fail**	0.209	320.7	56.76	54.35
	IL-38	0.540	**Fail**	0.527	57.64	51.53	47.83

AUC: area under the curve, %: percentage. Based on a rough classification system, AUC can be interpreted as follows: 90 -100 = excellent; 80 - 90 = good; 70 - 80 = fair; 60 - 70 = poor; 50 - 60 = fail [112].