

Salivary Neuropeptide Y, Interleukin-18, Tight Junction Proteins and Malondialdehyde as a Neuroimmune-Barrier Panel in Periodontitis

A Case-Control Study

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Abstract

Periodontitis involves interacting neural, immune, oxidative and epithelial barrier pathways, yet these are rarely measured together in the same patients. This cross-sectional case-control study evaluated a salivary panel comprising neuropeptide Y, interleukin-18, zonula occludens-1, claudin-2 and malondialdehyde in periodontitis. Forty non-smoking patients with periodontitis (Stage II, n = 20; Stage III–IV, n = 20) and 40 age- and sex-matched periodontitis-free controls were recruited at Tikrit Teaching Hospital, Iraq, between February and July 2026. Unstimulated whole saliva was analyzed by enzyme-linked immunosorbent assay, and diagnostic performance was assessed by receiver operating characteristic analysis with bootstrap internal validation, followed by multivariable logistic regression. All five biomarkers were 1.7- to 2.0-fold higher in periodontitis (all $P < 0.001$), remained higher after accounting for salivary flow ($P \leq 0.002$), increased with disease severity (P for trend < 0.001) and correlated with probing depth, attachment loss and bleeding on probing (Spearman coefficients 0.36–0.52). Individual areas under the curve ranged from 0.74 for claudin-2 to 0.81 for interleukin-18. The combined panel reached an area under the curve of 0.90 (95% confidence interval 0.83–0.97; optimism-corrected 0.88), exceeding interleukin-18 ($P = 0.012$) and malondialdehyde ($P = 0.006$). After adjustment for age, sex and plaque index, neuropeptide Y, interleukin-18 and malondialdehyde remained independently associated with periodontitis, whereas zonula occludens-1 and claudin-2 did not. A reduced panel of neuropeptide Y, interleukin-18 and malondialdehyde performed comparably to the full panel (area under the curve 0.88; $P = 0.284$). Salivary neuropeptide Y, interleukin-18 and malondialdehyde are independently associated with periodontitis, whereas salivary tight junction proteins track disease severity without independent association, consistent with downstream barrier injury.

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Introduction

Periodontitis is a chronic inflammatory disease of the tooth-supporting tissues that arises from

a dysregulated host response to a dysbiotic subgingival biofilm. It affects a large proportion of dentate adults worldwide and remains a leading cause of tooth loss [1,2]. Tissue

destruction in periodontitis is driven less by the microbial challenge itself than by a sustained, non-resolving host response in which innate immune activation, oxidative injury and

epithelial breakdown reinforce one another [3]. The 2017 World Workshop classification captured this heterogeneity through a staging and grading framework, yet staging still rests on clinical and radiographic measures of damage that has already occurred [4–6]. This reliance on cumulative clinical measures has encouraged the search for host-derived biomarkers that reflect current biological activity. Whole saliva is attractive for this purpose because it is collected non-invasively and integrates signals from all periodontal sites, the oral mucosa and the salivary glands [7]. Systematic reviews, however, indicate that individual salivary biomarkers show moderate and inconsistent diagnostic accuracy, whereas combinations of markers that represent different biological pathways tend to perform better [8,9]. The choice of pathways to combine is therefore a central design question.

One pathway that has received little attention in salivary studies is the neuroimmune axis. The gingiva is densely innervated by peptidergic fibers, and neuropeptides released from these fibers modulate vascular tone, leukocyte trafficking and cytokine production in the periodontium [10]. Neuropeptide Y (NPY), a 36-amino-acid peptide co-released with norepinephrine from sympathetic nerve terminals, acts on immune cells through Y1 receptors and exerts context-dependent pro- and anti-inflammatory effects [11,12]. Evidence in periodontitis is limited and not fully consistent: two gingival crevicular fluid (GCF) studies reported lower NPY at diseased sites than at healthy sites [13,14], whereas a salivary study found NPY to be higher in the saliva, but not the serum, of patients with periodontitis, where it correlated with disease extent, severity and bleeding on probing [15]. Salivary NPY has not, however, been examined together with inflammatory, oxidative and barrier markers in the same patients. Experimental work in the intestine has also shown that NPY interacts with tumor necrosis factor to regulate epithelial permeability [16], which raises the possibility that neural signals contribute to barrier failure in other mucosal sites.

The gingival epithelium forms the first physical barrier against the subgingival biofilm. Its integrity depends on intercellular junctions, including tight junctions composed of claudins, occludin and the cytoplasmic scaffold protein ZO-1 [17,18]. Periodontal pathogens can dismantle these structures directly: *Porphyromonas gingivalis* gingipains degrade junctional adhesion molecule 1 and permit the penetration of bacterial products into the connective tissue [19], and the same organism suppresses the epithelial transcription factor GRHL2, reducing junctional protein expression [20]. Tight-junction proteins also respond dynamically to

bacterial products in oral epithelial cells [21]. Claudin-2 is of particular interest because it forms cation-selective paracellular pores and is up-regulated by inflammatory cytokines, producing a leaky barrier in inflamed mucosa [22]. When junctional complexes are degraded or remodeled, fragments of these proteins may be released into the surrounding fluids, and their salivary concentration could therefore serve as an indirect readout of barrier injury. Two further processes link inflammation to barrier damage. IL-18 is a product of NLRP3 inflammasome activation, which is enhanced in periodontal tissues and saliva of patients with periodontitis [23–25]; a recent meta-analysis confirmed that IL-18 is elevated in periodontitis [26]. IL-18 also directly regulates epithelial barrier function in the intestinal mucosa [27]. Oxidative stress, conventionally measured through the lipid peroxidation product malondialdehyde (MDA), is well documented in periodontitis [28–30]. Reactive oxygen species disrupt epithelial tight junctions [31] and act as upstream signals for inflammasome activation [32], placing oxidative stress at the junction of the immune and barrier pathways.

Despite these mechanistic connections, neural, inflammasome, oxidative and barrier markers have generally been examined in isolation. The present study therefore measured a five-marker salivary panel comprising NPY, IL-18, ZO-1, claudin-2 and MDA in patients with periodontitis and periodontitis-free controls. The aims were to compare marker concentrations between groups, to examine their relationship with disease severity and clinical parameters, to evaluate the diagnostic performance of individual markers and combined panels, and to identify markers independently associated with periodontitis. We hypothesized that all five markers would be elevated in periodontitis and that a combined panel would discriminate disease more accurately than any single marker.

Material and Methods

This analytical cross-sectional case-control study was conducted at the outpatient clinics of Tikrit Teaching Hospital, Tikrit, Salah ad-Din Governorate, Iraq, between 1 February 2026 and 2 July 2026. Participants were recruited from individuals attending the outpatient clinics after verification of the inclusion and exclusion criteria. Clinical periodontal examination and saliva collection were performed on site, after which samples were transported to the laboratory for biochemical analysis. The study is reported in accordance with the STROBE guidelines for observational studies [33] and, for the diagnostic accuracy analyses, the STARD 2015 guidelines [34]. The study protocol was first

approved by the Scientific Affairs Division, College of Education for Pure Sciences, University of Samarra (letter no. 1647, 19 January 2026), and subsequently by the Scientific and Ethics Research Committee of the Salah ad-Din Health Directorate, Training and Human Development Centre (research project no. 2026-24; committee decision no. 24; session of 27 January 2026). Official permission to conduct the study at Tikrit Teaching Hospital was granted by the Research Management Unit, Salah ad-Din Health Directorate, Ministry of Health (letter no. 912, 27 January 2026). The study was conducted in accordance with the Declaration of Helsinki. Before enrolment, all participants were informed of the nature, aims and procedures of the study and provided written informed consent; confidentiality of personal data was assured by using coded identification numbers instead of names in clinical and laboratory records, and participants were free to withdraw at any time without any effect on the health care they received.

A total of 80 adults attending the outpatient clinics of Tikrit Teaching Hospital were enrolled after verification of eligibility and allocated to two equal groups: 40 patients with periodontitis and 40 periodontitis-free controls, frequency-matched for age and sex. Periodontitis was diagnosed according to the 2017 AAP/EFP classification on the basis of detectable interdental clinical attachment loss (CAL) with increased probing pocket depth (PPD) at multiple sites [4, 5]. For the secondary analysis, patients were staged according to the severity of interdental CAL, PPD and the extent of disease, with panoramic and, when needed, periapical radiographs used as an adjunct to assess alveolar bone loss and assign the stage; those with Stage II disease were classified as moderate ($n = 20$) and those with Stage III or IV disease as severe ($n = 20$), allowing a gradient to be examined from periodontal health through moderate to severe periodontitis. Controls showed no clinical evidence of periodontitis, defined as $PPD \leq 3$ mm and no interdental attachment loss attributable to periodontitis. Inclusion criteria were age 25 to 55 years and the presence of at least 20 natural teeth, excluding third molars, allowing a full-mouth periodontal examination. Exclusion criteria were current or former tobacco smoking; uncontrolled diabetes mellitus; systemic immune-mediated or inflammatory diseases; advanced renal or hepatic disease; malignancy; pregnancy or lactation; use of medications that may influence neural or immune responses, including β -blockers and certain psychotropic drugs; use of antibiotics or anti-inflammatory drugs within the preceding 3 months; and surgical or non-

surgical periodontal treatment within the preceding 6 months.

All clinical periodontal examinations were performed by a single trained and calibrated examiner. Before clinical data collection began, the examiner was trained and calibrated to standardize the periodontal examination and minimize measurement variability. Calibration was performed on 10 individuals who were not part of the study sample; PPD, CAL and BOP were recorded at the same sites on two occasions 7 days apart, with the second examination performed without reference to the first. Reproducibility of the continuous measurements was assessed separately for PPD and CAL with the intraclass correlation coefficient (ICC), and agreement in recording BOP, as a binary variable, with Cohen's kappa. Values ≥ 0.80 were predefined as acceptable; the ICC was 0.92 for PPD and 0.90 for CAL, and κ for BOP was 0.86, indicating high reproducibility before the main examination began.

All participants were then examined by the calibrated examiner using a mouth mirror and a UNC-15 periodontal probe. PPD, CAL and bleeding on probing (BOP) [35] were recorded at six sites per tooth (mesiobuccal, midbuccal, distobuccal, mesiolingual/palatal, midlingual/palatal and distolingual/palatal), together with the Plaque Index [36] and Gingival Index [37], on all teeth excluding third molars, using the same procedure for every participant. The clinical examination was carried out after saliva collection to avoid contamination of samples with blood. Unstimulated whole saliva was collected from all participants between 9:00 and 11:00 a.m. to limit diurnal variation in salivary secretion and biomarker levels. Participants were asked to refrain from eating, drinking (except water), tooth brushing and the use of mouthwash for at least 90 min before collection. After sitting in a comfortable position for several minutes, each participant provided saliva by the passive drool method [38] into a sterile tube over 5 min. Saliva was collected before the periodontal examination to avoid contamination with blood induced by probing. The volume of saliva and the collection time were recorded for each participant, and the unstimulated salivary flow rate was calculated as volume divided by collection time (mL/min).

Samples were transported to the laboratory under refrigeration and centrifuged at $3000 \times g$ for 15 min at 4°C to remove cells and debris. The clear supernatant was carefully collected, divided into separate aliquots and stored at -80°C until analysis; repeated freeze-thaw cycles were avoided. The maximum storage time before analysis did not exceed 3 months.

Salivary NPY, IL-18, ZO-1, claudin-2 and MDA were measured with commercial human ELISA kits (SunLong Biotech Co., Ltd., Hangzhou, China) according to the manufacturer's instructions. NPY was measured with the Human Neuropeptide Y (NPY) ELISA Kit (Cat. No. SL4604Hu; assay range 4.6–400 pg/mL; sensitivity 0.9 pg/mL; intra- and inter-assay coefficients of variation $< 10\%$ and $< 12\%$, respectively). IL-18 was measured with the Human Interleukin-18 ELISA Kit (Cat. No. SL0980Hu), ZO-1 with the Human ZO-1/TJP1 ELISA Kit (Cat. No. SL2819Hu), and claudin-2 with the Human Claudin-2 ELISA Kit (Cat. No. SL4486Hu). MDA was measured immunochemically with the Human Malondialdehyde ELISA Kit (Cat. No. SL1135Hu; assay range 7–400 ng/mL; sensitivity 1.5 ng/mL) rather than by the thiobarbituric acid reactive substances (TBARS) method. The assay ranges (sensitivities) were 1.5–100 pg/mL (0.5 pg/mL) for IL-18, 31.2–2000 pg/mL (6.1 pg/mL) for ZO-1, and 31.2–2000 pg/mL (5.6 pg/mL) for claudin-2. The intra- and inter-assay coefficients of variation were $< 10\%$ and $< 12\%$, respectively, for these assays and for MDA.

Reagents and samples were brought to the required temperature before each assay, and a separate standard curve was prepared for every run. Standards, samples and reagents were added following the manufacturer's sequence of steps. For NPY, samples were diluted 1:5 within the well (10 μL saliva plus 40 μL sample diluent). The IL-18 assay protocol likewise includes a 1:5 in-well dilution; samples whose in-well readings exceeded the upper limit of the standard curve (100 pg/mL) were further diluted 1:2 with sample diluent and re-assayed, giving a total dilution factor of 10, and final IL-18 concentrations were calculated by multiplying the values interpolated from the standard curve by this factor. Optical density was read at 450 nm with a BioTek ELx800 microplate reader (Agilent Technologies, Santa Clara, CA, USA), and concentrations were derived from the standard curve of each run, fitted with a five-parameter logistic (5PL) model for NPY, as recommended by the kit, and a four-parameter logistic (4PL) model for the other biomarkers. All samples were assayed in duplicate and the mean of the two readings was used. Samples whose readings fell outside the standard range were re-assayed after appropriate further dilution, and results were multiplied by the total dilution factor. All NPY measurements fell within the quantifiable range of the assay; a pre-specified rule of assigning LLOQ/2 to values below the lower limit of quantification was not required. Samples were labelled with sequential codes, laboratory personnel were blinded to the clinical classification of participants

throughout the measurements, and the code was broken only after the laboratory results had been finalized.

The sample size was calculated a priori with G*Power version 3.1.9.7 for a two-tailed comparison between two independent groups (t-test family). Assuming a medium-to-large effect size (Cohen's $d = 0.65$) based on preliminary pilot data obtained from a small sample before the study, $\alpha = 0.05$, power of 80% and a 1:1 allocation ratio, a minimum of 39 participants per group was required. Forty participants were enrolled in each group (total $n = 80$), which provides an achieved power of approximately 82% under the same assumptions.

Analyses were performed with IBM SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA); DeLong comparisons of AUCs and bootstrap internal validation were performed in R version 4.4.1 (R Foundation for Statistical Computing, Vienna, Austria). Normality was assessed with the Shapiro-Wilk test. Normally distributed variables are presented as mean $\pm$ standard deviation and compared with the independent-samples t-test; non-normally distributed variables are presented as median (interquartile range) and compared with the Mann-Whitney U test. Categorical variables were compared with the chi-square test. Differences across healthy, moderate and severe groups were assessed with the Kruskal-Wallis test followed by Dunn's post hoc test with Bonferroni correction, and ordered trends were evaluated with the Jonckheere-Terpstra test. Associations between biomarkers and clinical parameters were examined with Spearman's rank correlation in the whole cohort, and correlations among the five biomarkers were examined within the periodontitis group ($n = 40$) and, for comparison, in the whole cohort ($n = 80$).

Diagnostic performance was assessed by receiver operating characteristic (ROC) analysis. Optimal cut-offs were selected by maximizing the Youden index [39]. Combined panels were constructed from the predicted probabilities of logistic regression models, and correlated AUCs were compared with the DeLong test [40]. Optimism in the AUC of the combined five-marker panel was estimated by bootstrap resampling with 1000 replicates [41]. Independent associations were examined by multivariable logistic regression with all five biomarkers entered simultaneously and adjustment for age, sex and plaque index. Model calibration was assessed with the Hosmer-Lemeshow test, explained variance with Nagelkerke R^2 , and multicollinearity with variance inflation factors (VIF). To verify that group differences were not explained solely by differences in salivary dilution, the secretion rate of each

biomarker was calculated as its salivary concentration multiplied by the salivary flow rate and compared between groups in a secondary analysis with the Mann-Whitney U test. Two-sided P values < 0.05 were considered statistically significant.

Results

Of 112 individuals assessed for eligibility, 32 were excluded (5 did not meet the inclusion criteria, 20 met one or more exclusion criteria and 7 declined to participate), leaving 80 participants, all of whom were included in the final analysis (Figure 1). The two groups were comparable in age (39.2 ± 7.1 vs 40.0 ± 7.6 years; $P = 0.62$) and had identical sex distributions (Table 1). As expected, Plaque Index, Gingival Index, BOP, PPD and CAL were all significantly higher in the periodontitis group (all $P < 0.001$). Unstimulated salivary flow rate was slightly lower in patients than in controls, but the difference was not statistically significant (0.39 vs 0.46 mL/min; $P = 0.071$). Among patients, 20 had Stage II, 14 Stage III and 6 Stage IV periodontitis.

All five salivary biomarkers were significantly higher in patients with periodontitis than in healthy controls (all $P < 0.001$; Table 2). The largest relative increases were observed for NPY and MDA, whose median concentrations were approximately doubled (NPY 76 vs 38 pg/mL; MDA 43.6 vs 21.8 ng/mL). IL-18 and claudin-2 showed 1.9-fold increases, and ZO-1 showed a 1.74-fold increase. In the secondary analysis based on secretion rates (concentration $\times$ flow rate), all five biomarkers remained significantly higher in periodontitis ($P < 0.001$ for NPY, IL-18, claudin-2 and MDA; $P = 0.002$ for ZO-1), with fold differences of 1.49–1.71 (Supplementary Table S2). The group differences were therefore not explained by salivary dilution, although the difference for ZO-1 was somewhat attenuated.

When patients were stratified by stage, all biomarkers increased progressively from health through moderate to severe periodontitis (Kruskal-Wallis $P < 0.001$ and Jonckheere-Terpstra P for trend < 0.001 for every marker; Table 3). Both moderate and severe groups differed from healthy controls (all $P < 0.01$), and severe disease was associated with significantly higher concentrations than moderate disease for each marker. The separation between moderate and severe disease was most pronounced for MDA ($P = 0.008$) and IL-18 ($P = 0.012$), followed by NPY ($P = 0.021$), claudin-2 ($P = 0.027$) and ZO-1 ($P = 0.038$).

Every biomarker correlated positively and significantly with PPD, CAL and BOP ($r_s = 0.36$ – 0.52 ; Table 4). For each marker, the strongest correlation was with CAL. MDA and IL-18 showed the highest coefficients

overall (MDA-CAL $r_s = 0.52$; IL-18-CAL $r_s = 0.51$), whereas claudin-2 showed the weakest (claudin-2-BOP $r_s = 0.36$).

Within the periodontitis group, the strongest correlation was observed between the two tight-junction proteins, ZO-1 and claudin-2 ($r_s = 0.51$, $P < 0.001$; Table 5). IL-18 correlated with MDA ($r_s = 0.48$) and ZO-1 ($r_s = 0.42$) (both $P < 0.01$), and weakly with claudin-2 ($r_s = 0.36$). MDA correlated weakly with ZO-1 ($r_s = 0.39$) and claudin-2 ($r_s = 0.35$). NPY showed weak correlations with IL-18 ($r_s = 0.37$) and MDA ($r_s = 0.34$) (both $P < 0.05$) but was not significantly correlated with ZO-1 ($r_s = 0.29$, $P = 0.07$) or claudin-2 ($r_s = 0.25$, $P = 0.12$). In the whole cohort the same pattern was observed with stronger coefficients ($r_s = 0.31$ – 0.58 , all $P < 0.01$; Supplementary Table S1), reflecting the added between-group variation. No pair of markers exceeded $r_s = 0.6$ in either analysis.

ROC analysis showed fair to good discrimination for each marker (Table 6; Figure 2). IL-18 had the highest individual AUC (0.81; 95% CI 0.72–0.9), with a cut-off of ≥ 205 pg/mL giving a sensitivity and specificity of 77.5% each. MDA (AUC 0.8) and NPY (AUC 0.78) followed, while ZO-1 (0.75) and claudin-2 (0.74) performed least well.

The combined five-marker panel achieved an AUC of 0.9 (95% CI 0.83–0.97), with 85% sensitivity and 82.5% specificity at a predicted probability ≥ 0.48 . It outperformed the best single markers, IL-18 (Δ AUC = 0.09, 95% CI 0.02–0.16; DeLong $P = 0.012$) and MDA (Δ AUC = 0.1, 95% CI 0.03–0.17; $P = 0.006$). Bootstrap internal validation produced an optimism-corrected AUC of 0.88 (95% CI 0.8–0.95), indicating modest overfitting. A reduced panel restricted to NPY, IL-18 and MDA yielded an AUC of 0.88 (95% CI 0.8–0.95), with 82.5% sensitivity and 80% specificity, and did not differ significantly from the five-marker panel (DeLong $P = 0.284$).

In the multivariable logistic regression model adjusted for age, sex and plaque index, NPY (adjusted OR 1.31 per 10 pg/mL; 95% CI 1.02–1.69; $P = 0.036$), IL-18 (OR 1.08 per 10 pg/mL; 95% CI 1.02–1.15; $P = 0.013$) and MDA (OR 1.46 per 5 ng/mL; 95% CI 1.09–1.96; $P = 0.012$) remained independently associated with periodontitis (Table 7). ZO-1 showed a borderline association (OR 1.09; 95% CI 0.99–1.2; $P = 0.079$), and claudin-2 was not independently associated ($P = 0.112$). The model was well calibrated (Hosmer-Lemeshow $P = 0.621$), explained a substantial proportion of variance (Nagelkerke $R^2 = 0.53$), correctly classified 81.3% of participants and showed no relevant multicollinearity (maximum VIF = 2.18).

Discussion

This study examined, in the same individuals, salivary markers representing four interconnected processes in periodontitis: neural signalling (NPY), inflammasome activity (IL-18), oxidative injury (MDA) and epithelial barrier disruption (ZO-1 and claudin-2). All five markers were elevated in periodontitis, increased with disease stage and correlated with clinical severity. NPY, IL-18 and MDA were independently associated with periodontitis after adjustment for age, sex and plaque load, whereas the two tight-junction proteins were not. A combined panel discriminated periodontitis from health better than any single marker, and a reduced three-marker panel performed comparably to the full panel.

The NPY findings require careful interpretation. They agree with the only other salivary report we identified, in which NPY was higher in the saliva but not the serum of patients with periodontitis [15], but they differ in direction from earlier GCF studies. Lundy and colleagues reported higher NPY in GCF from healthy sites than from periodontitis sites and proposed a tonic, homeostatic role for the peptide [13], and a later split-mouth study likewise found lower GCF NPY in diseased periodontal and peri-implant sites [14]. Several factors may reconcile these observations with the salivary increase observed here. First, GCF reflects the local pocket environment, whereas whole saliva integrates contributions from all periodontal sites, the mucosa and the salivary glands, which receive sympathetic innervation; a glandular or mucosal increase in sympathetic output could therefore raise salivary NPY while local pocket concentrations fall, and the absence of a parallel serum increase in the earlier salivary study [15] points to a local rather than systemic source. Second, the protease-rich pocket environment may degrade or truncate NPY locally, reducing immunoreactive peptide in GCF without necessarily affecting whole-mouth concentrations to the same extent. Third, the earlier studies used different assay platforms, including radioimmunoassay, and antibody specificity for intact and truncated NPY forms may differ between methods. These explanations remain hypotheses, and paired GCF, saliva and plasma measurements would be needed to resolve them.

Irrespective of its source, the persistence of NPY as an independent correlate of periodontitis suggests that it is not simply a passive marker of inflammatory burden. NPY acting through Y1 receptors modulates macrophage and T-cell function in both stimulatory and inhibitory directions [11,12], and in the intestinal mucosa it amplifies tumor necrosis factor-driven inflammation and

increases epithelial permeability [16]. A comparable interaction in periodontal tissues would link sympathetic signaling to the barrier changes observed in this study. The correlation matrix supports this view: within the patient group, NPY was only weakly related to IL-18 and MDA and was not significantly correlated with either tight-junction protein, indicating that it carries information not captured by the inflammatory and barrier markers. The present design cannot establish a mechanism, but it identifies NPY as a candidate neuroimmune mediator worth investigating in periodontitis.

The elevation of salivary IL-18 agrees with previous reports of increased inflammasome activity in the saliva and serum of patients with periodontitis [24,42] and with a recent meta-analysis showing higher IL-18 levels in periodontitis across different biological fluids [26]. IL-18 had the highest individual AUC in our cohort and remained independent in the adjusted model. Beyond its role as an inflammatory cytokine, IL-18 regulates epithelial differentiation and barrier integrity, and excessive epithelial IL-18 signaling promotes barrier breakdown in experimental colitis [27]. This dual role makes IL-18 a plausible bridge between the inflammatory and barrier components of the panel.

MDA showed the strongest correlations with clinical parameters, particularly CAL, and the largest adjusted odds ratio. This is consistent with a meta-analysis showing elevated MDA in patients with periodontitis [30] and with the central role of neutrophil-derived reactive oxygen species in periodontal tissue destruction [28,29]. Reactive oxygen species disrupt tight-junction assembly and increase paracellular permeability in epithelial and endothelial cells [31], and oxidative stress is also an upstream trigger for NLRP3 activation and IL-18 maturation [32]. Oxidative stress may therefore link the inflammatory and barrier components measured in this study.

Both ZO-1 and claudin-2 were higher in the saliva of patients with periodontitis and rose significantly from moderate to severe disease. Because these are structural components of the junctional complex, their increase in saliva is best interpreted as release or shedding of junctional proteins from damaged or remodeling epithelium, not as a strengthening of the barrier. This interpretation fits experimental evidence that periodontal pathogens degrade junctional proteins and alter their expression in gingival epithelial cells [19–21]. The increase in claudin-2 is also biologically coherent: this pore-forming claudin is induced by inflammatory cytokines and mediates the leak pathway in inflamed mucosa [22]. Neither protein, however, remained independently associated

with periodontitis once NPY, IL-18, MDA and plaque index were included in the model. The simplest explanation is that salivary tight-junction proteins reflect a downstream consequence of inflammatory and oxidative injury, so that their information overlaps with that of the upstream markers. The inter-biomarker correlations are consistent with this: ZO-1 and claudin-2 formed the most closely related pair, as expected for co-released components of the same junctional complex, and both showed weak-to-moderate correlations with IL-18 and MDA, which were themselves correlated. Adjustment for plaque index may also have absorbed part of their association, because plaque accumulation is itself a driver of epithelial damage. Taken together, the data are compatible with a sequence in which neuroimmune and inflammasome activation, amplified by oxidative stress, leads to disruption of the epithelial barrier, although the cross-sectional design does not permit causal inference.

From a diagnostic perspective, the results agree with systematic reviews showing that combinations of salivary biomarkers outperform individual markers [8,9]. The five-marker panel improved significantly on IL-18 and MDA alone, and its optimism-corrected AUC of 0.88 indicates that over-fitting was limited. The reduced panel of NPY, IL-18 and MDA reached the same AUC with fewer assays, which favors it for further evaluation on grounds of cost and simplicity. Such a panel would not replace clinical probing, but it could support screening in settings where full periodontal examination is not routinely performed, or help monitor disease activity over time. These possibilities require confirmation in independent cohorts before any clinical use.

The main strengths of this study are the simultaneous measurement of neural, inflammatory, oxidative and barrier markers in the same participants, the exclusion of smokers, severity stratification according to the current classification, and a statistical approach that included DeLong comparisons, bootstrap internal validation and multivariable adjustment. Several limitations should also be acknowledged. The cross-sectional design precludes conclusions about temporal order or causality. The study was conducted at a single center with a modest sample; with 40 cases and eight variables in the adjusted model, the number of events per variable was below the commonly recommended threshold [43], so the regression estimates should be regarded as exploratory. The control group was defined by the absence of periodontitis rather than by strict periodontal health criteria, and some controls showed BOP values above 10%, consistent with localized gingival inflammation. Correlations

with clinical parameters were computed across the whole cohort, which combines two clinically distinct groups and may inflate the coefficients relative to within-group associations. Salivary concentrations were not normalized to total protein, although secretion rates adjusted for flow were examined in a secondary analysis, and ELISA detection of ZO-1 and claudin-2 in saliva cannot distinguish intact proteins from fragments. MDA was measured by ELISA rather than by the more widely used TBARS assay, which limits direct numerical comparison with many earlier periodontal studies. Finally, NPY was measured only in saliva, so its tissue source and relationship to GCF and circulating levels could not be determined. Longitudinal studies with paired sampling of saliva, GCF and plasma, and with assessment of the response to periodontal therapy, are needed to confirm these findings.

Conclusion

Salivary NPY, IL-18, ZO-1, claudin-2 and MDA were elevated in periodontitis and increased with disease severity. NPY, IL-18 and MDA were independently associated with periodontitis, whereas the salivary tight-junction proteins appeared to reflect downstream barrier injury. A combined neuroimmune-oxidative panel showed good discriminatory ability, and a parsimonious three-marker panel performed comparably to the full panel. These findings support further investigation of the neuroimmune-barrier axis in periodontitis and external validation of the proposed salivary panel.

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Author Contributions

All authors read and approved the final version of the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Use of AI-Assisted Tools

During the preparation of this manuscript, the authors used Claude (Anthropic) to assist with drafting, structuring and language editing of the text. The authors reviewed and edited all content and take full responsibility

for the data, analyses and conclusions reported.

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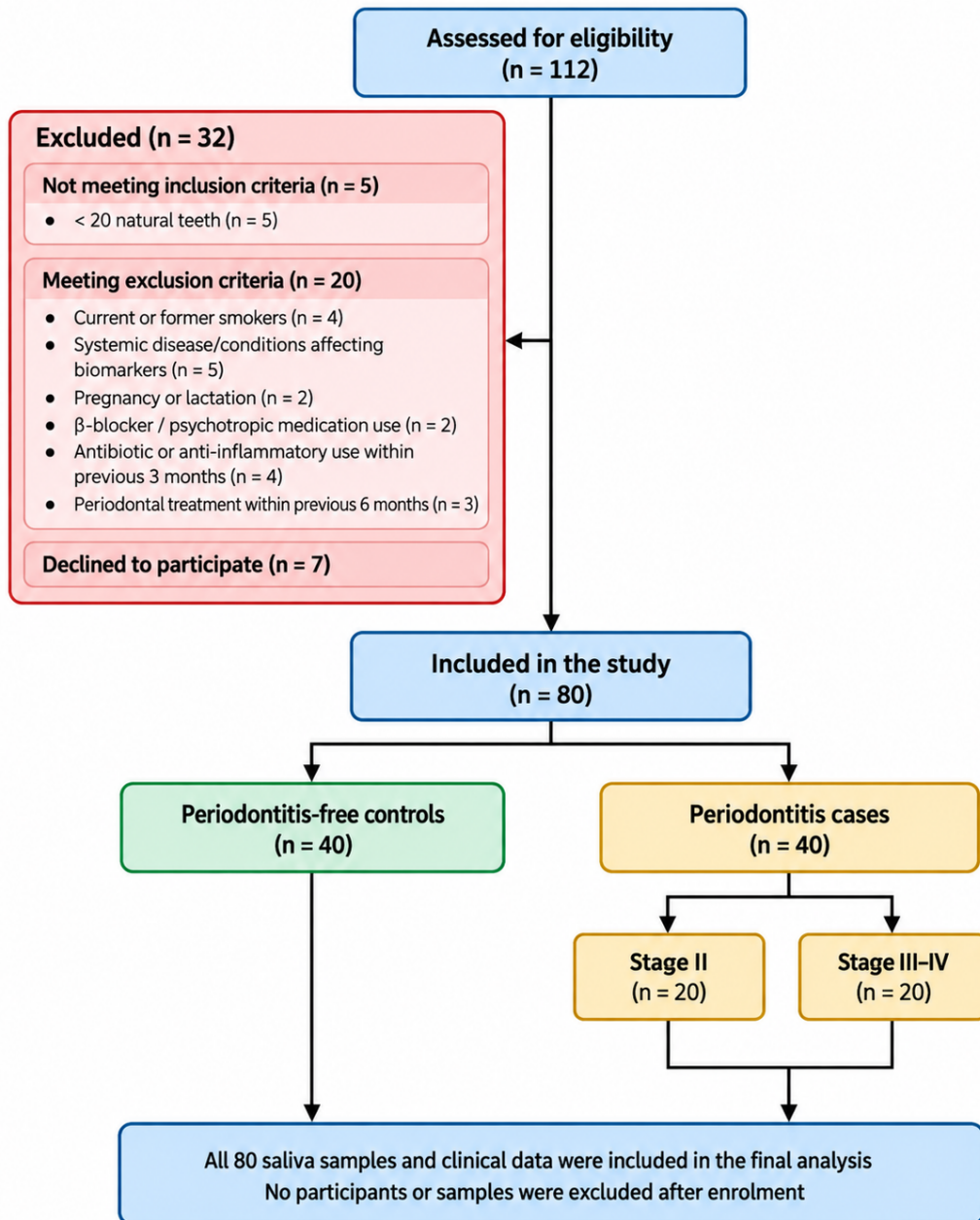


Figure 1. Flow of participants through the study according to the Standards for Reporting of Diagnostic Accuracy Studies 2015 guidelines. Of 112 individuals assessed for eligibility, 32 were excluded, and 80 participants were enrolled (40 periodontitis-free controls and 40 patients with periodontitis, subdivided into Stage II and Stage III-IV according to the 2017 World Workshop classification of periodontal diseases). No participant or sample was excluded after enrolment.

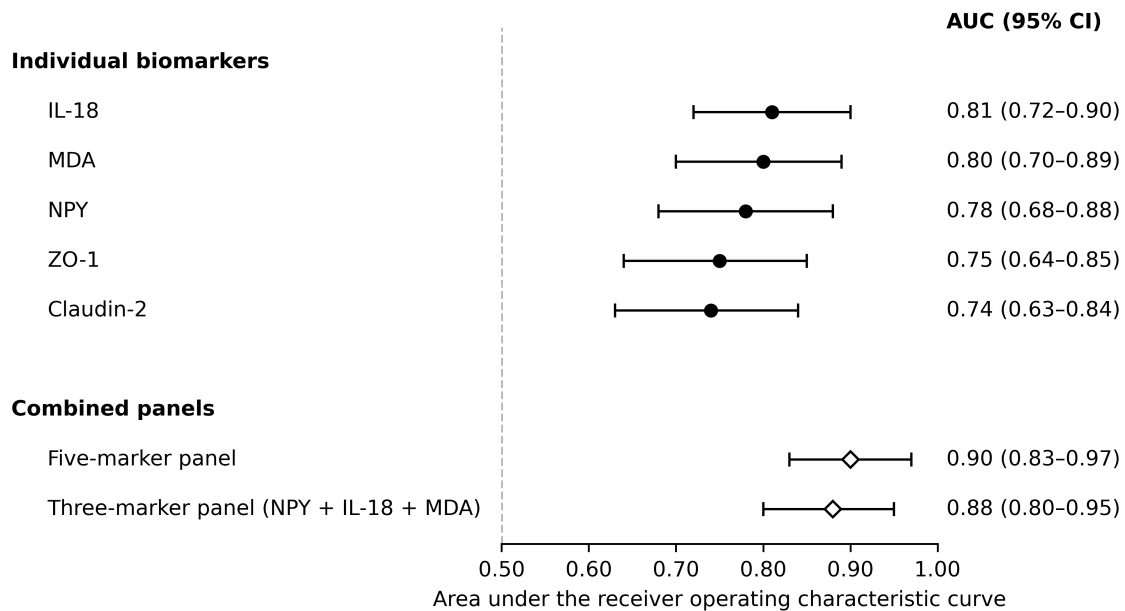


Figure 2. Diagnostic accuracy of individual salivary biomarkers and combined panels for discriminating periodontitis from periodontitis-free controls. Points indicate the area under the receiver operating characteristic curve (AUC) and horizontal bars its 95% confidence interval (CI); circles denote individual biomarkers and diamonds denote combined panels derived from multivariable logistic regression. The dashed vertical line marks chance discrimination (AUC = 0.50). The five-marker panel had a bootstrap optimism-corrected AUC of 0.88. IL-18, interleukin-18; MDA, malondialdehyde; NPY, neuropeptide Y; ZO-1, zonula occludens-1.

Table 1. Demographic and clinical characteristics of the study participants.

Variable	Controls (n = 40)	Periodontitis (n = 40)	P-value
Age (years)	39.2 ± 7.1	40.0 ± 7.6	0.62 ^a
Sex, male/female (n)	20/20	20/20	1.000 ^b
Plaque Index	0.61 ± 0.24	1.73 ± 0.41	<0.001 ^a
Gingival Index	0.46 ± 0.2	1.67 ± 0.38	<0.001 ^a
Bleeding on probing (%)	8 (5–12)	56 (45–68)	<0.001 ^c
Probing pocket depth (mm)	1.82 ± 0.3	5.08 ± 0.81	<0.001 ^a
Clinical attachment loss (mm)	0.2 (0–0.5)	4.3 (3.40–5.3)	<0.001 ^c
Unstimulated salivary flow rate (mL/min)	0.46 (0.38–0.56)	0.39 (0.31–0.48)	0.071 ^c
Stage II / III / IV (n)	—	20 / 14 / 6	—

Data are presented as mean ± SD or median (interquartile range). Current and former smokers were excluded. Controls were defined by the absence of periodontitis (see Methods). Periodontitis stage was assigned according to the 2017 AAP/EFP classification.

^a Independent-samples t-test; ^b Chi-square test; ^c Mann–Whitney U test.

Table 2. Salivary neuroimmune, epithelial barrier and oxidative stress biomarkers in periodontitis-free controls and patients with periodontitis.

Biomarker	Controls (n = 40)	Periodontitis (n = 40)	Fold change	Direction	P-value
NPY (pg/mL)	38 (27–52)	76 (54–105)	2	↑	<0.001
IL-18 (pg/mL)	145 (110–190)	275 (205–350)	1.9	↑	<0.001
ZO-1 (pg/mL)	95 (70–125)	165 (125–215)	1.74	↑	<0.001
Claudin-2 (pg/mL)	310 (225–430)	590 (415–810)	1.9	↑	<0.001
MDA (ng/mL)	21.8 (16.4–28.7)	43.6 (32.5–57.9)	2	↑	<0.001

Data are presented as median (interquartile range). P values were obtained with the Mann–Whitney U test. Fold change = median of the periodontitis group / median of the healthy group. All NPY measurements fell within the quantifiable range of the assay.

NPY, neuropeptide Y; IL-18, interleukin-18; ZO-1, zonula occludens-1; MDA, malondialdehyde.

Table 3. Salivary biomarker levels stratified by periodontitis severity.

Biomarker	Controls (n = 40)	Moderate (n = 20)	Severe (n = 20)	P (Kruskal–Wallis)	P for trend
NPY (pg/mL)	38 (27–52)	63 (48–79) ^a	91 (70–118) ^{ab}	<0.001	<0.001
IL-18 (pg/mL)	145 (110–190)	235 (190–285) ^a	315 (260–390) ^{ab}	<0.001	<0.001
ZO-1 (pg/mL)	95 (70–125)	145 (115–180) ^a	188 (150–235) ^{ab}	<0.001	<0.001
Claudin-2 (pg/mL)	310 (225–430)	505 (390–625) ^a	680 (520–860) ^{ab}	<0.001	<0.001
MDA (ng/mL)	21.8 (16.4–28.7)	35.2 (27.4–43.8) ^a	51.6 (40.2–65.9) ^{ab}	<0.001	<0.001

Data are presented as median (interquartile range). P for trend was calculated with the Jonckheere–Terpstra test. Pair-wise comparisons were made with Dunn’s test with Bonferroni correction.

^a P < 0.01 vs controls. ^b P < 0.05 vs moderate (NPY, P = 0.021; IL-18, P = 0.012; ZO-1, P = 0.038; claudin-2, P = 0.027; MDA, P = 0.008).

Moderate = Stage II; severe = Stage III–IV (2017 AAP/EFP classification).

Table 4. Spearman correlation coefficients between salivary biomarkers and clinical periodontal parameters (n = 80).

Biomarker	PPD, r _s	CAL, r _s	BOP, r _s
NPY	0.42***	0.46***	0.38***
IL-18	0.47***	0.51***	0.43***
ZO-1	0.40***	0.45***	0.38***
Claudin-2	0.38***	0.43***	0.36**
MDA	0.49***	0.52***	0.45***

r_s, Spearman’s rank correlation coefficient; PPD, probing pocket depth; CAL, clinical attachment loss; BOP, bleeding on probing. Correlations were computed in the whole cohort (healthy and periodontitis participants combined).

** P < 0.01; *** P < 0.001.

Table 5. Spearman correlation matrix among the five salivary biomarkers in patients with periodontitis (n = 40).

Biomarker	NPY	IL-18	ZO-1	Claudin-2	MDA
NPY	1.0				
IL-18	0.37*	1.0			
ZO-1	0.29	0.42**	1.0		
Claudin-2	0.25	0.36*	0.51***	1.0	
MDA	0.34*	0.48**	0.39*	0.35*	1.0

Values are Spearman's rank correlation coefficients (r_s). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 6. Diagnostic performance of individual salivary biomarkers and combined panels for discriminating periodontitis from periodontitis-free controls (ROC analysis).

Biomarker	AUC (95% CI)	Cut-off	Sensitivity (%)	Specificity (%)	Youden index	P-value
NPY	0.78 (0.68–0.88)	≥ 55 pg/mL	75	72.5	0.475	<0.001
IL-18	0.81 (0.72–0.9)	≥ 205 pg/mL	77.5	77.5	0.55	<0.001
ZO-1	0.75 (0.64–0.85)	≥ 125 pg/mL	72.5	70	0.425	<0.001
Claudin-2	0.74 (0.63–0.84)	≥ 430 pg/mL	70	70	0.4	0.001
MDA	0.8 (0.7–0.89)	≥ 30.5 ng/mL	77.5	75	0.525	<0.001
Combined 5-marker panel	0.9 (0.83–0.97)†	Predicted probability ≥ 0.48	85	82.5	0.675	<0.001
Reduced 3-marker panel (NPY + IL-18 + MDA)	0.88 (0.8–0.95)‡	Predicted probability	82.5	80	0.625	<0.001

AUC, area under the receiver operating characteristic curve; CI, confidence interval. Optimal cut-offs were determined by the maximum Youden index. Combined panels were built from the predicted probabilities of multivariable logistic regression models.

† Outperformed IL-18 (Δ AUC = 0.09, 95% CI 0.02–0.16; DeLong $P = 0.012$) and MDA (Δ AUC = 0.10, 95% CI 0.03–0.17; DeLong $P = 0.006$). Bootstrap optimism-corrected AUC (1000 resamples) = 0.88 (95% CI 0.80–0.95).

‡ Not significantly different from the 5-marker panel (DeLong $P = 0.284$).

Table 7. Multivariable logistic regression analysis of salivary biomarkers independently associated with periodontitis.

Variable	β	SE	Adjusted OR	95% CI	P-value
NPY (per 10 pg/mL)	0.27	0.129	1.31	1.02–1.69	0.036
IL-18 (per 10 pg/mL)	0.077	0.031	1.08	1.02–1.15	0.013
ZO-1 (per 10 pg/mL)	0.086	0.049	1.09	0.99–1.2	0.079
Claudin-2 (per 50 pg/mL)	0.113	0.071	1.12	0.97–1.29	0.112
MDA (per 5 ng/mL)	0.378	0.151	1.46	1.09–1.96	0.012

β , regression coefficient; SE, standard error; OR, odds ratio; CI, confidence interval. All five biomarkers were entered simultaneously; the model was adjusted for age, sex and plaque index.

Model fit: Hosmer–Lemeshow $\chi^2 = 6.24$, $df = 8$, $P = 0.621$; Nagelkerke $R^2 = 0.53$; overall classification accuracy = 81.3%; maximum VIF = 2.18 (IL-18). Bold values indicate $P < 0.05$.

Supplementary Table S1. Spearman correlation matrix among the five salivary biomarkers in all participants (n = 80).

Biomarker	NPY	IL-18	ZO-1	Claudin-2	MDA
NPY	1.0				
IL-18	0.44***	1.0			
ZO-1	0.36**	0.49***	1.0		
Claudin-2	0.31**	0.42***	0.58***	1.0	
MDA	0.41***	0.55***	0.46***	0.43***	1.0

Values are Spearman's rank correlation coefficients (r_s). ** $P < 0.01$; *** $P < 0.001$. Coefficients in the whole cohort include between-group variation and are therefore larger than those within the periodontitis group (Table 5).

Supplementary Table S2. Salivary biomarker secretion rates (concentration $\times$ flow rate) in periodontitis-free controls and patients with periodontitis.

Biomarker	Controls (n = 40)	Periodontitis (n = 40)	P-value
NPY (pg/min)	17.6 (12.1–24.8)	29.4 (20.3–41.7)	<0.001
IL-18 (pg/min)	66.3 (49.2–88.5)	106.8 (78.6–142.7)	<0.001
ZO-1 (pg/min)	43.5 (31.1–59.7)	64.7 (47.6–86.9)	0.002
Claudin-2 (pg/min)	141.8 (102.6–198.4)	228.9 (164.3–317.6)	<0.001
MDA (ng/min)	10 (7.3–13.8)	17.1 (12.2–23.6)	<0.001

Data are presented as median (interquartile range). Secretion rate = salivary concentration $\times$ unstimulated salivary flow rate (mL/min). P values were obtained with the Mann–Whitney U test.