# Salivary and Gingival Crevicular Fluid Levels of Interleukin-1β, Interleukin-17, Interleukin-36β, Interleukin-37 and Interleukin-38 In Healthy and Periodontitis Patients: A Cross-sectional Study

A Thesis

Presented to the Faculty of Tufts University School of Dental Medicine

in Partial Fulfillment of the Requirements for the Degree of

Master of Science in Dental Research

by

**Reem Mohammad Alreshaid, BDS**

June 2024

Tufts University School of Dental Medicine

Department of Periodontology

Boston, MA

# THESIS COMMITTEE MEMBERS

## Principal Investigator

**Evangelos Papathanasiou, DDS, MS, DMD, PhD, MS**
Associate Professor,
Department of Periodontology,
Tufts University School of Dental Medicine

## Co-Investigators

**Mabi Singh, DMD, MS**
Professor
Department of Diagnostic Sciences - Oral Medicine
Tufts University School of Dental Medicine

**Livia Valverde, DMD, MS, PhD, DMSc**
Assistant Professor, Predoctoral Director
Department of Periodontology,
Tufts University School of Dental Medicine

**Matthew Finkelman, BA, PhD**
Associate Professor
Director of Biostatistics and Experimental Design
Public Health and Community Service
Tufts University School of Dental Medicine

## ABSTRACT

**Aim & Hypothesis:** To compare the levels of IL-1β, IL-17, IL-36β, IL-37 and IL-38 in gingival crevicular fluid (GCF) and saliva collected from periodontitis patients and healthy subjects. The hypothesis of the study was that the levels of IL-1β, IL-17, IL-36β, IL-37 and IL-38 in GCF and saliva would be different in periodontitis patients compared to healthy subjects.

**Materials & Methods:** In this cross-sectional study, clinical data, GCF and saliva samples were collected from 40 participants, divided into healthy and periodontitis groups based on specific selection criteria. The samples were analyzed by a blind assessor using Luminex multiplex technology to quantify the levels of IL-1β, IL-17, IL-36β, IL-37, and IL-38. Statistical analyses included chi-square or Fisher's exact tests for categorical variables and t-tests or Mann-Whitney U tests for continuous variables. Generalized linear models (GLMs) adjusted for age, gender and calculated saliva weight, and generalized estimating equations (GEE) were used for correlated data.

**Results:** GCF levels of IL-1β and IL-17, both amount and concentration, were significantly elevated in periodontitis patients ($p < 0.001$). Conversely, IL-36β concentration in GCF was reduced in periodontitis patients. Additionally, the concentrations of IL-37 and IL-38 in GCF were markedly lower in periodontitis patients ($p=0.046$, $p=0.012$ respectively). Salivary IL-1β concentration was also

significantly higher in periodontitis patients (p= 0.005).

**Conclusions:** Our study supports our hypothesis that periodontitis patients have distinct IL-1β, IL-17, IL-36β, IL-37, and IL-38 GCF and salivary profiles compared to periodontally healthy subjects. Further studies are needed to confirm if IL-1β, IL-17, IL-36β, IL-37, and IL-38 can be used as diagnostic markers and therapeutic targets for periodontitis.

# DEDICATION

To my beloved parents, Mohammad and Moudhi. Your unwavering encouragement, love, and sacrifices have given me the strength, resilience, and dedication needed to complete this journey. You are the foundation of all my accomplishments, and I am eternally grateful.

To my extraordinary siblings, Khwlah, Khalid, Sultan, Khoolod, and Abdulrahman. Thank you for your constant support and belief in me, and all the joy you bring.

To my mentor, Dr. Papathanasiou. Your rigorous standards and profound expertise have inspired me to excel. I deeply appreciate your guidance and mentorship.

To my committee members, Dr. Finkelman, Dr. Valverde, and Dr. Singh, for their invaluable advice and assistance throughout this process.

To the Department of Periodontology for their ongoing support.

To all my friends, thank you for being by my side.

# ACKNOWLEDGMENTS

We thank the Multiplex Core at The Forsyth Institute (Cambridge, MA), particularly Ms. Michelle Patel, for leading all multiplex protein analysis services. We also extend our gratitude to the Department of Periodontology and the Department of Research for providing the necessary funds to support the clinical study and the analysis of the samples. Special thanks to all the subjects who participated in the study. Additionally, we acknowledge the Department of Research Administration for their support in regulating all documents for IRB approval.

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## 1.1 Periodontitis

Periodontal disease is an infectious inflammatory disease of the periodontium associated with dental bacterial plaque and, if left untreated, can lead to tooth loss [1,2]. The prevalence of periodontitis in US adults over the age of 30 is considered high, affecting (~46%) around 64.7 million adults, categorizing periodontitis as the 6$^{th}$ most chronic human disease and a significant public health concern [3,4]. Periodontitis is characterized by gingival inflammation, periodontal pocket depth (PD) formation, bleeding on probing (BOP), clinical attachment loss and alveolar bone resorption [5]. Several factors contribute to the etiopathogenesis of periodontitis initiated by the accumulation of bacterial plaque that triggers the host immune/inflammatory response responsible for the periodontal tissue destruction [6,7]. Innate immunity is the first line of host defense against periodontal pathogens that plays a crucial role in attempting to contain the periodontal infection or in propagating further periodontal tissue destruction. Its' response to the pathogenic bacteria is facilitated by the local secretion of cytokines from several host immune cells such as macrophages, neutrophils, and lymphocytes[8]. Cytokines are known as low molecular weight proteins that function as inflammatory mediators and regulate the extent and the period of the host response [9].

Dysbiosis, also known as dysregulated host immune response or microbial community imbalance, can result in disruption within the periodontium that results in loss of the supporting bone and degraded extracellular matrix [10]. Interleukin-1 (IL-1) Cytokine family has both pro-inflammatory and anti-inflammatory properties. The pro-inflammatory ILs include IL-1α, IL-1β, IL-18, IL-33, IL-36, while the anti-inflammatory ILs include IL-1 receptor antagonist (IL-1Ra), IL-36Ra, IL-37, and IL-38 [6]. The destructive actions of the pro-inflammatory cytokines are supposed to be controlled by anti-inflammatory cytokines that attempt to reduce the progression

of periodontal disease[11]. The regulation of inflammatory mediators by endogenous mechanisms and the balance between pro- and anti-inflammatory mediators determines the severity of tissue destruction.

IL-1beta (IL-1β), Tumor necrosis factor-α (TNF-α), IL-6, and Receptor activator of nuclear factor-kappa-B ligand (RANKL) have the most substantial evidence among cytokines functioning in networks [12]. The first cytokines involved in the early stages of the pathogenesis of periodontal disease are TNF-α, IL-1β, and IL-6. The alveolar bone destruction is the outcome of periodontal cells stimulated by the mediators of inflammatory cells to secrete matrix metalloproteinases (MMPs) that are proteolytic enzymes breaking down the connective tissue and contribute to bone loss. Prostaglandins (PGs) are lipid mediators of inflammation. The production of a potent alveolar bone resorption stimulator Prostaglandin $E_2$ ($PGE_2$) by periodontal ligament cells is enhanced by cytokines of the innate immune response including IL-1β, TNF-a, and parathyroid hormone [13]. According to the Seventh European Workshop on Periodontology Field Consensus, the Osteoprotegerin (OPG) to RANKL ratio (OPG/RANKL) seems to be a valuable biomarker test for bone loss [12].

Periodontal disease diagnostic tests can be developed with the use of oral fluids, which are easily collected and contain locally and systemically derived biomarkers that may help develop tools for predicting the periodontal status of an individual [14,15]. Gingival crevicular fluid (GCF) is an inflammatory exudate as well as a physiological fluid, and its composition includes tissue breakdown products, inflammatory mediators, and antibodies. The composition of GCF is used prospectively to identify subclinical changes, thus serving a diagnostic purpose [14].

Saliva contains different biomolecules that aid in detecting early oral and systemic diseases, monitoring treatment outcomes and contributing to disease prognosis by developing distinct biomarkers. Different salivary biomarkers investigated in the oral, pancreatic, lung, and breast cancers reported having a potential for detecting cancer [16]. In systemically healthy subjects, matrix metalloproteinase-8 (MMP-8), MMP-9, IL-1β, and IL-6 salivary biomarkers resulted in a good capability to identify periodontitis, as concluded in a recent systematic review [17]. Nevertheless, literature is limited on IL-1 family members in saliva and their role in periodontal disease. This could be due to the methodological challenges in detecting and quantifying these cytokines in saliva. [17]

## 1.2 IL-1β

IL-1β is a pro-inflammatory cytokine that plays a key role in periodontal tissue destruction [6]. Monocytes/macrophages and dendritic cells (DCs) secrete IL-1β in response to other pro-inflammatory cytokines such as TNF-α and in response to several pathogen-associated molecular patterns (PAMPs). IL-1β activates IL-receptor, which will lead to the recruitment of other molecules and ultimately trigger a greater magnitude of inflammatory genes[6,18]. IL-1β has been considered a strong GCF biomarker for periodontitis severity, disease progression, adverse outcomes, and treatment [19]. A systematic review by Stadler et al. aimed to compare the levels of Cytokines/Chemokines in GCF between chronic periodontitis and healthy patients. The pro-inflammatory cytokines IL-1β, IL-6, interferon-gamma (IFN-γ), and monocyte chemoattractant protein-1 MCP-1/ CCL2 chemokine levels in GCF were significantly higher in patients with chronic periodontitis compared to healthy [20]. IL-1β has a role in the differentiation of T cells to

the major source of IL-17, T helper-17 (Th17) cells, and Toll-like receptor ( TLR) activation, which will result in the IL-37 secretion [6,21].

## 1.3 IL-17

IL-1 has a key role in the differentiation of T cells toward IL-17-producing cells that have an essential role in several inflammatory and autoimmune diseases [22]. As a pro-inflammatory cytokine, IL-17 is critical in host defense against microbial infections and is found to be associated with many autoimmune disorders, metabolic disorders, and cancer [21]. IL-17 was found to be higher in GCF and blood collected from periodontitis patients compared to healthy subjects and concluded its prominent role in periodontal inflammation [23]. The role of salivary IL-17 in periodontitis is still not well understood, as different studies show varying results regarding its levels in periodontitis patients compared to healthy subjects. The study by Vahabi et al found higher salivary IL-17 concentrations in healthy subjects compared to chronic periodontitis patients[24]. In contrast, another study by Yang et al found significantly higher levels of salivary IL-17 in periodontitis patients compared to healthy subjects[25].

## 1.4 IL-36β

IL-36 and its subfamily (IL-36α, IL-36β, IL-36γ, and IL-36Ra ) are pro-inflammatory cytokines mainly expressed by epithelial cells, fibroblasts, and keratinocytes. IL-36 cytokines have been investigated recently for their role in autoimmune diseases, including psoriasis, rheumatoid arthritis (RA), inflammatory bowel disease (IBS), systemic lupus erythematosus (SLE), neuromyelitis optica spectrum disorder, primary Sjögren's syndrome (pSS), Myasthenia gravis and systemic sclerosis [26]. They have a role in regulating the inflammation of the mucosa

and hemostasis by amplifying the production of antimicrobial peptides and cytokines [6]. The first study demonstrated the presence of IL-36γ, IL-36β, and IL-33 in GCF samples done by Kurşunlu et al. [27]. In this study, patients with generalized aggressive periodontitis had higher GCF levels of IL-36β than patients with chronic periodontitis. In contrast, gingivitis patients had similar IL-36β levels as healthy subjects, suggesting that increased GCF levels of IL-36β might be related to the severity of the periodontitis [27]. More studies are needed to investigate the expression and secretion of IL-36 cytokines in GCF and saliva samples in periodontitis patients to better understand their role in the pathogenesis of periodontal diseases [6].

## 1.5 IL-37

IL-37 is an anti-inflammatory cytokine produced by macrophages after IL-1β and transforming growth factor-β (TGF-β) activates Toll-like Receptors (TLRs). IL-37 inhibits IL-18, a proinflammatory cytokine, from binding to IL-18Rα [6]. It is considered unique because of its role in suppressing both innate and acquired immunity [28]. Patients with inflammatory diseases such as SLE, IBS, psoriasis, atherosclerosis, pSS, and rheumatoid arthritis (RA) have increased IL-37 mRNA production. In contrast, the severity of certain diseases such as allergic rhinitis, asthma, and insulin resistance showed reduced levels of IL-37, which may reveal a failure in fighting inflammation [28].

A genome-wide association study investigated the role of IL-37 and revealed that the downgrade of IL-37 led to the upregulation of IL-1β highlighting the possible anti-inflammatory role of IL-37 in periodontitis [29]. However, the role of IL-37 in its periodontal disease is still limited, and more studies are needed.

Another study compared the levels of IL-37 in GCF, saliva, and plasma in healthy and periodontally diseased subjects [30]; IL-37 was detected in all biofluids. However, the authors concluded that IL-37 is not a useful diagnostic marker for distinguishing periodontitis from health [30]. There is limited evidence of the role of IL-37 in periodontal disease, and its' role needs to be investigated further.

## 1.6 IL-38

IL-38 is a newly discovered cytokine. It is secreted by various immune cells, mainly B cells, and expressed in the heart, thymus, kidney, and skin. Different expression levels of IL-38 were found in different autoimmune diseases such as RA, SLE, psoriasis, and irritable bowel syndrome [31]. IL-38 is similar to IL-36Ra, presenting its antagonistic effect through its binding to IL-36 Receptor. The dose-response curve of IL-38 is like that of IL-37. It was found that low levels of IL-38 have higher potency in blocking IL-17 candida-induced responses, while high levels of IL-38 increased IL-17 secretion, resembling IL-37 characteristics. The biology of IL-38 is not yet meticulously comprehended [32].

The ability of IL-38 to inhibit the cell response of T helper 17 (Th17) was studied in Primary Sjogren's Syndrome pathophysiology, knowing that pSS patients presented higher levels of IL-17 secreted from Th17. The study involved collecting blood and saliva samples from minor salivary gland mononuclear cells of pSS patients and comparing them to controls. The results presented low IL-38 and higher Th17 cytokine expression in patients with pSS than controls in both serum samples, concluding the inhibition of Th17 response by IL-38[33].

The only study that investigated the role of IL-38 in oral fluids and serum was published recently [34]. The authors concluded that IL-38 amount in GCF increases as the severity of

periodontitis increases and IL-38 could be possibly used to determine the activity of periodontal disease [34].

The blood serum levels of IL-38 were higher in patients with untreated or early multiple sclerosis and systemic sclerosis than in treated patients and healthy participants in a sex-matched cross-sectional study [35]. Further studies are needed to investigate the role of IL-38 cytokines in the pathogenesis of oral and periodontal diseases.

## 1.7 GCF/Saliva Biomarkers and Periodontal Diseases

Current clinical diagnostic parameters for periodontitis include various periodontal measurements such as PD, BOP, clinical attachment level (CAL), plaque index (PI), and alveolar bone levels radiographically. However, all these periodontal measurements reflect historical activity of periodontitis but not current disease activity and they do not allow us to identify susceptible individuals who might be at risk of future periodontitis progression [36]. Dental medicine needs a more precise diagnostic tool that acts as “point of care” and facilitates earlier diagnosis, accurate prognosis, and assessment of periodontal therapeutic outcomes. Identifying patients at risk for periodontal destruction progression is a challenge for clinicians, and developing a new periodontal diagnostic tool with biomarkers using GCF and saliva can facilitate earlier diagnosis and improve the management of periodontal disease[37,38].

Kornman stated that stratifying patients into specific well-defined categories is the first step in personalized periodontal medicine [37]. Identifying periodontitis biomarkers from an easily accessible diagnostic oral fluid has the benefit of distinguishing patients that require personalized preventive care and customized treatment protocol,leading to less invasive and lower-cost

treatments and enhancing the outcome by controlling the progression of the periodontal disease [39].

## 1.8 Aims and Hypotheses

The aim of this cross-sectional study was to compare the levels of IL-1β, IL-17, IL-36β, IL-37 and IL-38 in GCF and saliva collected from periodontitis patients and healthy subjects.

The hypothesis of the study was that the levels of IL-1β, IL-17, IL-36β, IL-37 and IL-38 in GCF and saliva would be different in periodontitis patients compared to healthy subjects. Specifically, we hypothesize that the levels of IL-1β, IL-17, and IL-36β would be higher in periodontitis patients compared to healthy subjects and that the levels of IL-37 and IL-38 would be lower in periodontitis patients compared to healthy subjects.

# 2. Materials and Methods

## 2.1 Study population

We conducted a cross-sectional study involving 40 participants (n=40) divided equally into two groups (n=20 subjects/group): 20 periodontally healthy subjects (Group A) and 20 periodontitis patients (Group B). Volunteers were recruited and enrolled from the Department of Periodontology and other clinics at Tufts University School of Dental Medicine. The study protocol was reviewed and approved by the Tufts University Health Sciences Institutional Review Board (IRB) prior to subjects' enrollment (00002786).

## 2.2 Inclusion and exclusion criteria

We used the following criteria to enroll the study subject to all groups:

1. Participants between the age of 18 – 75 years old.
2. Having at least 20 natural teeth in their dentition – excluding third molars.
3. In good systemic health.

Specific inclusion criteria for the periodontitis group:

1. BOP ≥ 20 % of probing sites.
2. ≥ 5 teeth with at least one site each with PD ≥5 mm, CAL >3mm and BOP.
3. Radiographic indication of interproximal bone loss >2mm, measured from the cemento-enamel junction (CEJ) to the bone crest at sites with PD ≥5 mm and BOP.

Based on the new classification introduced by the American Academy of Periodontology (AAP) and the European Federation of Periodontology (EFP) in 2017, patients who are diagnosied with either Stage 3 or stage 4 periodontitis were enrolled in this study.[40]

Specific inclusion criteria for the periodontally healthy group:

1. BOP< 10% of the surfaces in total.
2. Absence of PD > 4 mm.
3. Absence of CAL > 4 mm.
4. Radiographic evidence of interproximal bone level at a distance of ≤ 2 mm, measured from the CEJ to the bone crest.

We also applied the following exclusion criteria:

1. Systemic disease (self-reported) that has an impact on periodontal status (e.g., diabetes mellitus, HIV/AIDS, rheumatoid arthritis).
2. Self-reported history of any autoimmune disease (e.g., pSS, SLE, psoriasis, IBS).
3. Self-reported history of immunosuppressive chemotherapy or history of any disease known to severely compromise immune function (e.g., cytomegalovirus infection) within the last 5 years.
4. Any acute intraoral infection (e.g., herpetic gingivostomatitis, herpangina, abscesses).
5. Need for antibiotic premedication for routine dental procedures.
6. Use of antibiotics within the last 3 months.
7. Self-reported daily use of steroids or non-steroid anti-inflammatory therapy within the last 3-months.

8. Patients who self-report pregnancy or lactation (to avoid any radiographs on pregnant women, also hormonal changes that can affect periodontal health and aggravate existing periodontal disease).
9. Inability or unwillingness to sign an informed consent form. Evaluated by the PI/Co-Is.
10. Current smokers.
11. Previous periodontal treatment (even prophylaxis) in the past 3 months prior to the examination.
12. Anything that would place the individual at increased risk or preclude the individual's full compliance with or completion of the study at the discretion of the investigators. Evaluated by the PI/Co-Is.
13. Non-English speakers.
14. Pregnant individuals.

Written informed consent was obtained from all participants after reviewing the study protocol in detail with each subject and giving them ample time to study and think, and answer any of their questions.

## 2.3 Clinical examination

### 2.3.1 Single-Visit Study (50-70 minutes):

Informed consents were obtained by the investigator (PI/Co-Is). The subjects were asked to read the informed consent form (ICF), given ample time to have any questions answered, and then asked to sign the ICF. The subjects were given a copy of the ICF. The subjects were asked to

complete demographic information and a medical history. The subjects were asked about any family history of periodontitis.

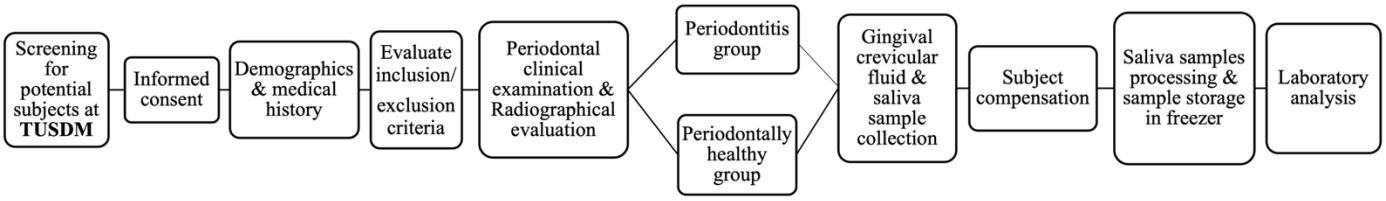
An oral exam, including evaluation of oral cavity soft and hard tissues, was completed following standard of care procedure in US dentistry using a mouth mirror and dental explorer/probe. Inclusion/exclusion criteria were evaluated.

The study visit procedures and the estimated time of the single visit study are shown in Table 1. The study schema is depicted in Figure 1.

Table 1. The study visit procedure and the estimated time.

| Appointment / Procedures | Visit 1 | Estimated time |
|---|---|---|
| Informed Consent Form | X | 30-40 minutes |
| Demographics and medical history | X | |
| Evaluate eligibility and withdrawal criteria | X | |
| Periodontal clinical examination and radiographical evaluation | X | |
| Acquisition of Gingival crevicular fluid and saliva sample | X | 2-15 minutes |
| Payment | X | 1 minute |

Figure 1: Study Schema.



### 2.3.2 Periodontal clinical examination:

Clinical parameters were recorded at the same visit by one experienced periodontal resident (RA) and verified by the PI at six periodontal sites (mesiobuccal, buccal, distobuccal, distolingual/distopalatal, lingual/palatal, and mesiolingual/mesiopalatal) of all teeth, excluding third molars.

Parameters include the following: plaque index (PI), gingival index (GI), Bleeding on Probing (BOP) within 15 seconds, suppuration on probing within 15 seconds (SUB), and mobility (MOB) scored according to Table 2. Probing Depth (PD); Recession (REC); Clinical Attachment Level (CAL), were measured and recorded to the nearest millimeter (mm). Parameters were determined using a University of North Carolina periodontal probe (PCP-UNC 15; Hu-Friedy Manufacturing Company, Chicago, III).

Table 2. Scoring of indices.

| | 0 | 1 |
|---|---|---|
| Plaque Index | Plaque not present | Plaque present |
| Gingival Index | Gingival redness not present | Gingival redness present |
| Bleeding on Probing | No bleeding within 15 seconds of probing | Bleeding within 15 seconds of probing |
| Suppuration on Probing | No suppuration within 15 seconds of probing | Suppuration within 15 seconds of probing |
| Mobility | Not present | Present |

### 2.3.3 Radiographic evaluation:

The same examiner (RA) who performed the clinical examination also conducted a radiographic evaluation of any interproximal bone loss for each patient based on radiographs that had already been taken for each patient. In the case of absence of any radiographs exposed over 12 months before, new radiographs were taken upon the discretion of the examiner. It was the study team's clinical judgment that it is standard of care to use recent radiographs (no older than 12 months) and to take new radiographs if recent ones do not exist and are necessary to verify our diagnosis and stored in a patient's dental record. Any new radiographs taken for the research study were at no cost to the patient or the patient's dental insurance. Only recent radiographs can provide high diagnostic quality for accuracy in the diagnosis of periodontal diseases, especially in distinguishing periodontitis from gingivitis (based on the presence/absence of interproximal bone loss). Clinical judgment was used as to the need for and type of radiographic images for evaluation and/or monitoring of periodontal disease.

The above-mentioned clinical and radiographic examination is part of the standard comprehensive periodontal examination of any patient who is screened and treated in the Department of Periodontology or the Undergraduate Clinic at Tufts University School of Dental Medicine, Boston, MA, for accurate diagnosis of periodontal disease. The radiographs were taken as standard of care and added to the subjects' dental records.

During screening, subjects were asked to bring in existing radiographs that were less than one year old if they had them. If they were of good diagnostic quality, they were used. If not, new ones were taken following the standard of care (SOC) procedures outlined above.

Subjects were then divided into periodontally healthy or periodontitis subject groups following the above criteria.

## 2.4 Gingival crevicular fluid (GCF) sampling

GCF samples were collected from two sites on two different teeth (one site from each tooth, N=2 GCF samples per subject) and pooled in the same Eppendorf tube that was coded and labeled.

In healthy subjects, the GCF samples were randomly collected from one site on one maxillary posterior tooth (premolar/molar area) and one site on one mandibular posterior tooth (premolar/molar area).

In periodontitis subjects, GCF samples were collected from two sites of two different teeth (one site from each tooth, N=2 GCF samples per subject), pooled in the same coded labeled Eppendorf tubes. The sites with the deepest PDs were selected; in cases of similar PDs, the most accessible site from posterior teeth (premolar/molar area) from the maxilla/mandible was selected.

GCF sample collection started with removing first supragingival plaque with a curette. Then, the area was isolated with cotton rolls and air-dried. Periopaper strips (Periopaper, Interstate Drug Exchange, Amityville, NY, USA) were inserted gently 1-2 mm into the opening of the sulcus subgingivally and left in place for 30 seconds. Blood or saliva visibly contaminated periopaper was discarded following proper discarding procedures. GCF volume was determined from each periopaper strip using Periotron 8000 (Oraflow), which was calibrated based on protocol previously described [41]. Referring to the standard calibration curve, the readings were converted to an actual volume (µl) by using the device's software (Periotron MLCONVRT).

Two periopaper strips (N=2 GCF samples per subject) were immediately placed in coded labeled Eppendorf tubes after measuring the GCF volume with Periotron device and stored at -80 °C until they were transported to the Forsyth Institute for further laboratory cytokine analysis

(one transportation). Proper storage and handling procedures were followed by the study team as outlined in the IBC registration. A log (Excel Spreadsheet) of samples placed in the freezer was continuously updated by the study team [42].

## 2.5 Saliva samples

### 2.5.1 Saliva samples collection

First, the samples' vial weight (gm) was measured before and after saliva collection using Ohaus Adventurer SL Precision Balance with Round Draftshield (AS-153). The participants were asked to provide saliva samples. Unstimulated whole saliva with a sample volume range of 1-5 ml was collected from all subjects by sitting quietly and drooling into a cup for 5 minutes after initial swallowing. Saliva samples vials were then placed in ice and transported to the PI's laboratory for further centrifuging and processing.

### 2.5.2 Saliva Samples Processing

The samples were aliquoted into 1 ml solutions from the whole saliva collected placed in different Eppendorf tubes. Samples were centrifuged in coded labelled tubes, maintaining the temperature at 4°C for 20 min at a force of 12,000 relative centrifugal forces (rcf) using Eppendorf centrifuge 5415 D machine. The supernatant was transferred in new Eppendorf tubes with coded labels and stored in the PI's laboratory freezer (-80 °C) until transported to the Forsyth Institute for further laboratory cytokine analysis. A log (Excel Spreadsheet) of samples placed in the freezer was updated continuously.

## 2.6 Cytokines Quantification

Levels of IL-1β, IL-17, IL-36β, IL-37, and IL-38 were determined using a multiplexed sandwich immunoassay analysis performed by the Multiplex Core Facility at the Forsyth Institute (Cambridge, MA) based on the flowmetric LuminexTM xMAP technology. The kits used were Millipore Human High Sensitivity T Cell Panel (Millipore - HSTCMAG-28SK) and Millipore Human Cytokine/Chemokine IV Panel (Millipore - HCYP4MAG-64K-03). GCF samples were vortexed and centrifuged at 14,500 g for 5 minutes.

A 12.5 µL aliquot from each sample was combined with a 12.5 µL 1:500 dilution of a protease inhibitor cocktail (Millipore Corporation). The values were calculated to the final sample dilution.

The saliva samples were liquified and centrifuged at 9300g for 5 minutes at room temperature. Salivary IL-1β, IL-17, IL-36β, IL-37 and IL-38 concentrations were analyzed using the LuminexTM xMAP technique at the Forsyth Institute (Cambridge, MA).

The amount of a cytokine refers to the total quantity present in each sample of GCF, measured in picograms (pg). The concentration is the amount of the cytokine per unit volume, expressed in picogram per microliter (pg/µL) for GCF and picogram per milliliter (pg/mL) for salivary samples. The cytokine concentration provides information on the cytokine levels present in each volume.

The amount (pg) and concentrations of the cytokines from both GCF (pg/µl) and saliva (pg/mL) samples were recorded. GCF concentration was calculated by dividing the amount of each cytokine (pg) by the volume of each sample (µl). The laboratory technicians provided the results in an Excel sheet.

The Pro-inflammatory index is a measure used to assess the level of inflammatory mediators in a sample. It was calculated by adding the amounts (pg) of IL-1β, IL-17, and IL-36β, and then dividing this sum by the volume (µl for GCF and ml for saliva samples). This calculation gives the concentration of pro-inflammatory mediators in the sample. The Anti-inflammatory index, on the other hand, measures the concentration of anti-inflammatory mediators in a sample. It was calculated by adding the amounts (pg) of anti-inflammatory cytokines, IL-37 and IL-38, and then dividing this sum by the volume (µl of GCF and ml for saliva samples) .

Luminex technology utilizes fluorescently dyed microspheres that bind to specific antibodies targeting various cytokines, generating unique spectral signatures for each cytokine. Samples are analyzed using a flow cytometry-like system, where lasers excite the dyes, and the fluorescence intensity is measured to accurately quantify the cytokines [43].

## 2.7 Statistical Analysis:

Based on data from previously published studies [27,30], a power calculation was performed using nQuery (nQuery Advisor, 7.0). For IL-37, a calculation was made assuming a Type I error rate of 5% and a Type II error rate of 20%. The assumed mean ± SD GCF concentration was 19.86±16.02 pg/µl for healthy subjects, while the assumed mean ± SD IL-37 GCF concentration was 5.01±7.61 pg/µl for subjects with periodontitis. The minimum sample size was calculated to be 13 subjects per group for IL-37 [30].

For IL-36β, a sample size calculation was made assuming a Type I error rate of 5% and a Type II error rate of 20%. The assumed mean ± SD GCF concentration was 78.15±47.22 pg/µl for healthy subjects, and the assumed mean ± SD IL-36β GCF concentration was 35.16±25.21

pg/µl for subjects with periodontitis. The minimum sample size was calculated to be 14 subjects per group for IL-36β [27].

To have a more robust sample for our study, the sample size was increased to 20 subjects per group for a total of 40 participants, which represents 94.99% and 92.62% power for IL-37 and IL-36β, respectively.

Demographic and clinical data were analyzed using counts and percentages for categorical variables and means, standard deviations (SDs), medians, and inter-quartile ranges (IQRs) for continuous variables. To compare two groups in terms of categorical variables, the chi-square test or Fisher's exact test was used. Depending on the assumption of normality (which was assessed using the Shapiro-Wilk test), the independent-samples t-test or Mann-Whitney U test was used to compare two groups in terms of continuous variables in analyses of independent data. Multivariable modeling was also conducted using generalized linear models (GLMs) to adjust for age, gender, and saliva weight; the Akaike information criterion (AIC) was used to specify the distribution family in the GLMs. For analyses with two sites per subject, generalized estimating equations (GEE) were used to account for the presence of correlated data within a subject. The Spearman correlation was used to evaluate associations between continuous variables. All tests of significance were two-sided, and the level of significance was set at $p<0.05$. The analysis was performed using SPSS 28 (IBM Corp., Armonk, NY, USA) and SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

## 2.8 Data Imputation

We used data imputation method to replace the missing values with one-fifth of the minimum detected value for each interleukin in order to address missing data. We used a

recognized data imputation method commonly applied in proteomic analysis for datasets that include missing values [44]. This common method ensures that imputed values are based on the minimum observed value and fall within the range of the dataset [45,46].

# 3. RESULTS

## 3.1 Demographics and Clinical parameters

Table 3. presents the mean demographic and clinical parameters for both study groups (healthy and periodontitis). Each group included 20 subjects. The mean ± SD age in the healthy group was 51.40±12.13 years, and the mean ± SD age in the periodontitis group was slightly lower, at 50.40±12.7 years. The difference in age between the two groups was not statistically significant ($p= 0.801$) as determined by the independent-samples t-test.

The majority of healthy patients were female (65%), while the majority of periodontitis patients were male (55%). The groups had a similar mean number of missing teeth (1.45±1.80, 1.45±2.00, respectively). The difference in gender distribution and the number of missing teeth was not statistically significant ($p = 0.204$ and $p= 0.490$, respectively) as determined by the chi-square test for gender and the Mann-Whitney U test for number of missing teeth.

Significant differences were observed in all measured clinical parameters PD, CAL, BOP, PI, SUB, MOB between healthy and periodontitis patients determined by the Mann-Whitney U test.

PD and CAL were higher in the periodontitis group compared to the healthy group ($p<0.001$). There was a notable difference in PI, with healthy individuals at 16.18% compared to 59.23% in the periodontitis group ($p<0.001$).

In the periodontitis group, BOP was significantly higher at 48.55% as compared to the healthy group's 2.01% ($p<0.001$). No suppuration was found in the healthy group, but it was detected in 4.44% of cases from the periodontitis group ($p<0.001$). The rate of mobility rose significantly to 20.67% for the periodontitis group and was significantly different from that of healthy group which stood at 2.18% ($p<0.001$).

Ethnicity and race of the participants are summarized in Table 3. Overall, most of the participants were not Hispanic or Latino and identified as White in both groups. No individuals of Native Hawaiian/Pacific Islander or American Indian/Alaskan descent reported in either group.

Table 3. Demographic and clinical parameters for periodontally healthy and periodontitis subjects.

| Mean (± SD)<br>Median ($25^{th}$ – $75^{th}$ percentiles) | Healthy (n = 20) | Periodontitis (n = 20) | P-value | |
|---|---|---|---|---|
| **Age** | 51.40±12.13<br>12.13(42.0-63.80) | 50.40±12.73<br>50.50(42.25-59.50) | 0.801[a] | |
| **Gender** | | | | |
| **% Male (N)** | 35% (7) | 55% (11) | 0.204[b] | |
| **% Female (N)** | 65% (13) | 45% (9) | | |
| **Number of missing teeth** | 1.45±1.80<br>1.00(0.00-2.80) | 1.45±2.00<br>0.00(0.00-3.50) | 0.749[c] | |
| **Probing depth (mm)** | 2.39±0.26<br>2.42(2.22-2.54) | 3.69±0.84<br>3.50(3.18-4.09) | **<0.001**[*c] | |
| **Clinical Attachement level (mm)** | 0.69±0.85<br>0.20(0.036-1.21) | 3.76±1.66<br>3.53(2.85-4.37) | **<0.001**[*c] | |
| **PI ( % )** | 16.18±19.07<br>11.80(0.00-25.90) | 59.23±35.95<br>56.10(28.30-100) | **<0.001**[*c] | |
| **BOP ( % )** | 2.01±2.40<br>1.23(0.60-2.44) | 48.55±36.74<br>44.20(17.04-83.66) | **<0.001**[*c] | |
| **Suppuration ( % )** | 0.00<br>0.00 | 4.44±9.23<br>0.30(0.00-6.10) | **<0.001**[*c] | |
| **MOB ( % )** | 2.18±7.25<br>0.00 | 20.67±21.50<br>14.60(0.90-32.14) | **<0.001**[*c] | |
| | | | | |
| **% Ethnicity (N)** | | | **Total** | **P-value** |
| **Not Hispanic or Latino** | 90% (18) | 80% (16) | 85% ( 34) | 0.695[d] |
| **Hispanic or Latino** | 5% (1) | 10% (2) | 7.5% (3) | |
| **Unknown/Not reported** | 5% (1) | 10% (2) | 7.5% (3) | |
| **% Race (N)** | | | | |
| **White** | 65% (13) | 50% (10) | 57.5% (23) | 0.088[d] |
| **Black/African American** | 0% | 25% (5) | 12.5% (5) | |
| **Native Hawaiian/Pacific Islander** | 0% | 0% | 0% | |
| **American Indian/Alaskan** | 0% | 0% | 0% | |
| **Asian** | 20%(4) | 15%(3) | 17.5%(7) | |
| **Multiple** | 0% | 5.0% (1) | 2.5%(1) | |
| **Don't Know** | 0% | 0% | 0% | |
| **No Answer/Other** | 15%(3) | 5.0% (1) | 10% (4) | |

* Statistically significant difference (P < .05).

a Determined by Independent-samples t-test.

b Determined by Chi-square test.

c Determined by Mann-Whitney U test.

d Determined by Fisher's exact test.

## 3.2 Data for groups of sites of GCF collection

The clinical parameters of sites used for GCF sampling for healthy and periodontitis patients are displayed in Table 4. Fourty-two samples were collected from the maxillary arch and 38 samples were collected from the mandibular arch following our previously described methodology.

Probing depths mean ± SD PD between healthy groups' sites was 2.85±0.36 mm, while in periodontitis sites mean ± SD PD was 7.57±1.44 mm. For sites with periodontitis, mean ± SD CAL was 7.90±2.19 mm, while in healthy group mean ± SD CAL was 0.57±1.15 mm.

Sites of GCF collection in healthy subjects had 25% PI, while in periodontitis patients PI was higher and scored 70%. BOP was 100% for periodontitis patients and 0% in healthy ones
No suppuration was noted in healthy subjects, while 10% of sites had suppuration among periodontitis patients. Mobility was also not found among sites of GCF collection in healthy subjects, while 30% of sites of GCF collection in periodontitis patients displayed mobility

Statistically significant differences were found across all parameters between sites of GCF collection in healthy and periodontitis patients. The p-values for PD, CAL, and % BOP were found to be less than 0.0001, while the p-value for PI was 0.005, as determined by generalized estimating equations (GEE).

Table 4. Clinical parameters for all groups of sites of GCF.

| | | Healthy (n = 20) | Periodontitis (n = 20) | Total |
|---|---|---|---|---|
| **Arch:** | | | | |
| **Maxillary** | | 20 | 22 | 42 |
| **Mandibular** | | 20 | 18 | 38 |
| | | | | **P- Value** |
| **PD (mm)** | Mean (SD) | 2.85±0.36 | 7.57±1.44 | **<0.001**[*a] |
| | Median (25th - 75th percentiles) | 3.00 (3.00-3.00) | 7.00 (6.00-9.00) | |
| **CAL (mm)** | Mean (SD) | 0.57±1.15 | 7.90±2.19 | **<0.001**[*a] |
| | Median (25th - 75th percentiles) | 0.00 (0.00-0.75) | 8.00 (6.00-9.75) | |
| **PI ( % )** | | 25% | 70% | **0.005**[*a] |
| **BOP ( % )** | | 0% | 100% | **<0.001**[*a] |
| **Suppuration ( % )** | | 0% | 10% | **<0.001**[*a] |
| **MOB ( % )** | | 0% | 30% | **<0.001**[*a] |

* Statistically significant difference (P < .05).

a Determined by Generalized estimating equations (GEE)

## 3.3 Data for GCF volume

The GCF volume measurements for the two groups are shown in Table 5. For healthy subjects, the mean ± SD GCF volume was 1.21±0.58 ul. For periodontitis patients, the mean ± SD GCF volume was 2.11±0.5 ul. The difference in GCF volume between healthy individuals and those with periodontitis was statistically significant ($p<0.001$) as determined by the Mann-Whitney U test. Figure 2 depicts GCF volume levels in both studied groups.

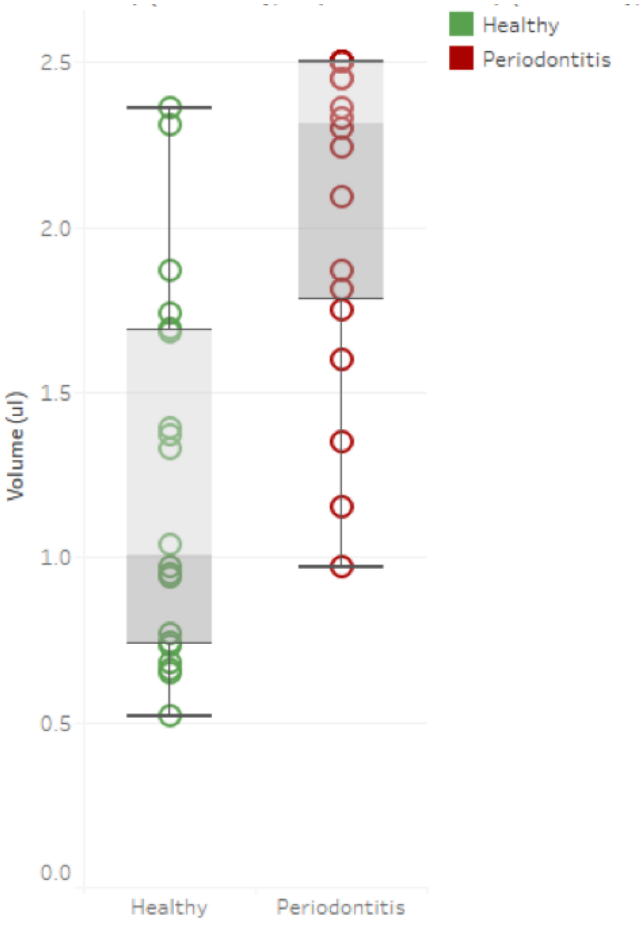
Table 5. GCF volume mean levels ±SD for all groups of GCF collection.

| | Healthy (n = 20) | Periodontitis (n = 20) | P-value |
|---|---|---|---|
| **GCF volume** | | | |
| Mean ± SD | 1.20±0.58 | 2.11±0.50 | **<0.001*c** |
| Median (25th - 75th percentiles) | 1.00 (0.73-1.70) | 2.31 (1.80-2.50) | |

* Statistically significant difference (P < .05).

c Determined by Mann-Whitney U test.

Figure 2. Box-plot of GCF volume of healthy and periodontitis subjects.



## 3.4 Frequency of detection of inflammatory cytokines

The percentage of sites testing positive for detection of IL-1β, IL-17, IL-36β, IL-37β, and IL-38 in GCF and saliva between healthy individuals and periodontitis patients are shown in Tables 6 and 7 along with their associated p-values determined with Fisher's exact test. Figures 3 and 4 are bar charts illustrating the frequency of detection in both studied groups.

- **GCF**

IL-1β and IL-36β were positive in all GCF samples (100%) in both the healthy and periodontitis groups. IL-17 was detected in only 50% of the healthy group's GCF samples compared to 100% detection in periodontitis patients ($p<0.001$). IL-37 was detected in 95% of both the healthy and periodontitis groups, with no statistically significant difference ($p=1.000$). IL-38 was detected in 85% of the sites of GCF samples in healthy subjects, while in a lower number of samples (60%) in GCF samples of periodontitis patients ($p=0.150$).

- **Saliva**

IL-1β and IL-36β were detected in all healthy and periodontitis patients (100%), similar to GCF samples' frequency detection. IL-17 had a low detection frequency of only 20% in both the healthy and periodontitis groups.

IL-37 was found in a high frequency of detection (95%) in both the healthy and periodontitis groups. IL-38 was detected in 50% of salivary samples in periodontitis patients but more frequently in saliva of healthy subjects (75%) following a similar trend as in GCF detection. No statistically significant difference was found in the detection frequency of inflammatory cytokines in saliva between healthy and periodontitis patients.

Since the detection frequency of IL-17, IL-37, and IL-38 were not 100% in GCF and saliva, we used data imputation method by replacing the missing values with 1/5 of the minimum detected level of each interleukin.

Table 6: Frequency of detection of IL-1β, IL-17, IL-36β, IL-37, IL-38 in GCF.

| % sites (N) positive for | Healthy (n = 20) | Periodontitis (n = 20) | P-Value |
|---|---|---|---|
| **IL-1β** | 100% (20) | 100% (20) | 1.000[d] |
| **IL-17** | 50% (10) | 100% (20) | **<0.001**[*d] |
| **IL-36β** | 100% (20) | 100% (20) | 1.000[d] |
| **IL-37** | 95% (19) | 95% (19) | 1.000[d] |
| **IL-38** | 85% (17) | 60% (12) | 0.150[d] |

* Statistically significant difference (P < .05).

d Determined by Fisher's exact test.

Table 7: Frequency of detection of IL-1β, IL-17, IL-36β, IL-37, IL-38 in Saliva.

| % sites (N) positive for | Healthy (n = 20) | Periodontitis (n = 20) | P-Value |
|---|---|---|---|
| **IL-1β** | 100% (20) | 100% (20) | 1.000[d] |
| **IL-17** | 20% (4) | 20% (4) | 1.000[d] |
| **IL-36β** | 100% (20) | 100% (20) | 1.000[d] |
| **IL-37** | 95% (19) | 95% (19) | 1.000[d] |
| **IL-38** | 75% (15) | 50% (10) | 0.190[d] |

* Statistically significant difference (P < .05).

d Determined by Fisher's exact test.

Figure 3: Bar chart of frequency of detection of IL-1β, IL-17, IL-36β, IL-37, IL-38 in GCF.

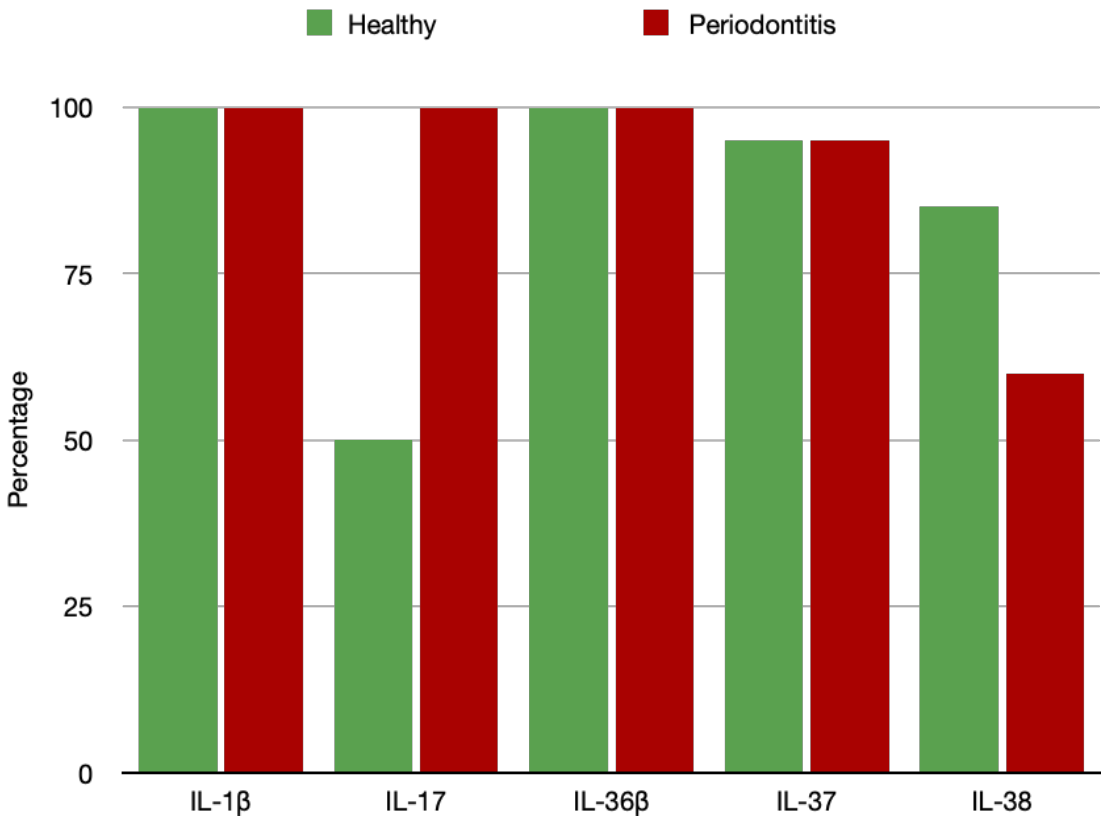
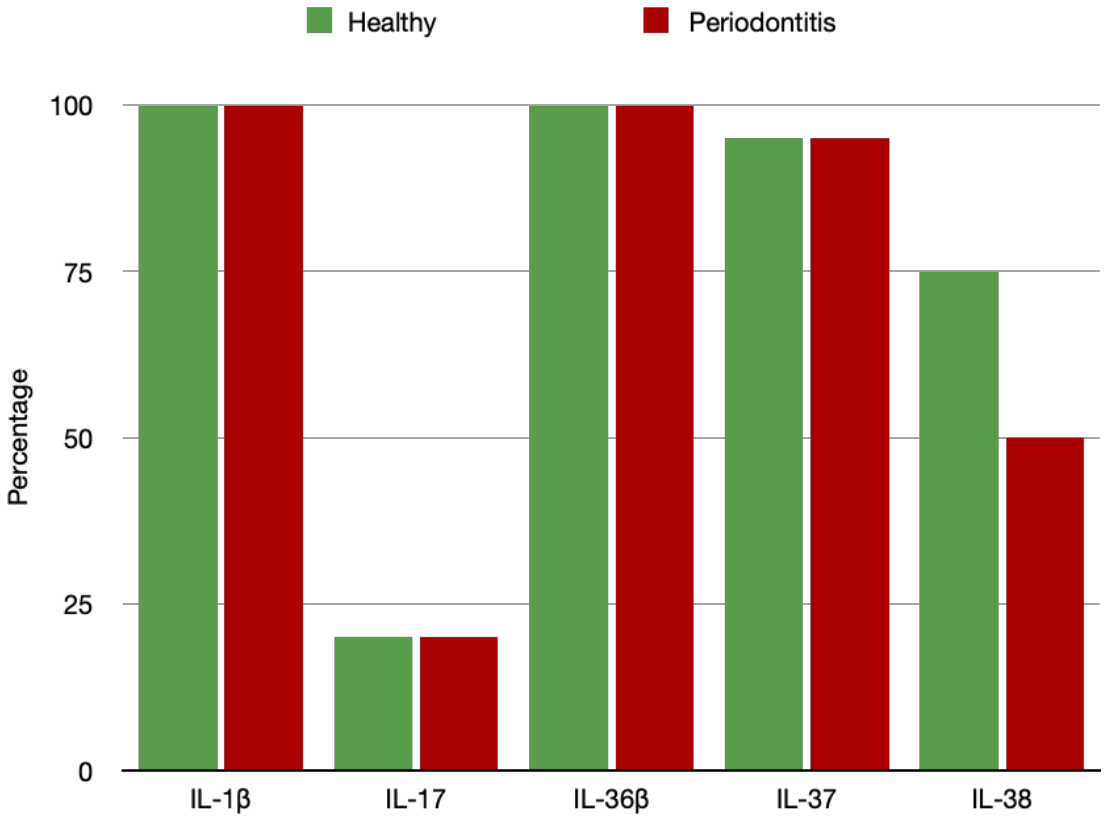


Figure 4: Bar chart of frequency of detection of IL-1β, IL-17, IL-36β, IL-37, IL-38 in saliva.



## 3.5 Inflammatory Mediators' Levels in GCF

Tables 8 and 9 present the levels of inflammatory mediators in GCF samples (amount and concentration) collected from both groups (healthy and periodontitis). Figures 5 and 6 are bar charts depicting the amounts and concentrations of interleukins in both groups. For IL-1β, IL-17, IL-37, and IL-38 amount and concentration data the Shapiro-Wilk test indicated that the data did not follow a normal distribution. Therefore, the Mann-Whitney U test was used to assess statistical significance between the two groups. Regarding IL-36β concentration, the Shapiro-Wilk test indicated that our data did not follow a normal distribution. Therefore, the Mann-Whitney U test was used to assess statistical significance between the two groups. IL-36β amount data did not exhibit evidence of non-normality after conducting the Shapiro-Wilk test and an independent-samples t-test was accordingly used.

- **Amount (pg) of inflammatory mediators in GCF**

We first compared the amount levels of inflammatory mediators between healthy and periodontitis patients without adjusting covariates (age and gender). IL-1β and IL-17 amount (pg) were significantly higher in periodontitis patients compared to healthy subjects ($p < 0.001$). IL-36β amount levels were higher in the periodontitis group compared to the healthy group. IL-37 amount levels were higher in healthy subjects compared to periodontitis patients, while IL-38 showed similar levels in both groups. For IL-36β, IL-37, and IL-38 there was no significant difference in GCF amount between the healthy and periodontitis groups.

There was no difference for amount levels of inflammatory mediators in GCF when we adjusted for age and gender compared to the results before adjusting for the covariates (age and gender).

- **Concentration levels (pg/ul) of inflammatory mediators in GCF**

The concentration was calculated by dividing the amount (pg) of each sample by GCF volume (ul).

GCF concentration of IL-1β and IL-17 was significantly higher in periodontitis subjects compared to healthy subjects ($p<0.001$). These differences also remained significant after adjustment for gender and age ($p<0.001$ and $p=0.002$, respectively).

IL-36β showed a significantly higher GCF concentration in healthy subjects compared to periodontitis patients ($p=0.049$). However, the difference for IL-36β did not remain significant after adjusting for age and gender ($p =0.088$).

GCF concentration of IL-37 was significantly higher in healthy individuals compared to periodontitis patients ($p=0.046$; $p=0.017$ after adjustment).

IL-38 concentration was significantly higher in healthy individuals compared to periodontitis patients ($p=0.012$), which remained significant after adjusting for age and gender ($p = 0.011$).

GCF concentrations of all interleukins in both healthy and periodontitis patients are illustrated in box-plots seen in Figure 7.

Table 8: GCF amount (pg) of IL-1β, IL-17, IL-36β, IL-37, IL-38.

| Interleukin | | Healthy | Periodontitis | P-value |
|---|---|---|---|---|
| **IL-1β** | Mean (SD) | 44.20±59.06 | 383.22±388.8 | **<0.001**[*a] |
| | Median (25th - 75th percentiles) | 20.02 (5.88-49.41) | 284.8 (159.59-449.09) | **<0.001**[*b] |
| **IL-17** | Mean (SD) | 0.42±0.50 | 2.75±1.84 | **<0.001**[*a] |
| | Median (25th - 75th percentiles) | 0.19 (0.058-0.67) | 2.50 (1.11-3.92) | **<0.001**[*b] |
| **IL-36β** | Mean (SD) | 184.45±89.28 | 224.18±99.08 | 0.191[a] |
| | Median (25th - 75th percentiles) | 174.82 (121.1-212.2) | 219.8 (132.79-304.31) | 0.157[b] |
| **IL-37** | Mean (SD) | 62.63±70.06 | 57.93±52.16 | 0.889[a] |
| | Median (25th - 75th percentiles) | 49.8 (22.91-72.82) | 40.05 (20.48-88.73) | 0.455[b] |
| **IL-38** | Mean (SD) | 10.77 ± 6.94 | 11.36 ±12.17 | 0.611[a] |
| | Median (25th - 75th percentiles) | 10.58 (6.33-15.2) | 7.76 (0.78-20.2) | 0.269[b] |

* Statistically significant difference (P < .05).

a Determined by Mann-Whitney U test

b Adjusted for age and gender

Figure 5: Bar chart of amount (pg) of IL-1β, IL-17, IL-36β, IL-37, IL-38 in GCF of healthy and periodontitis subjects.

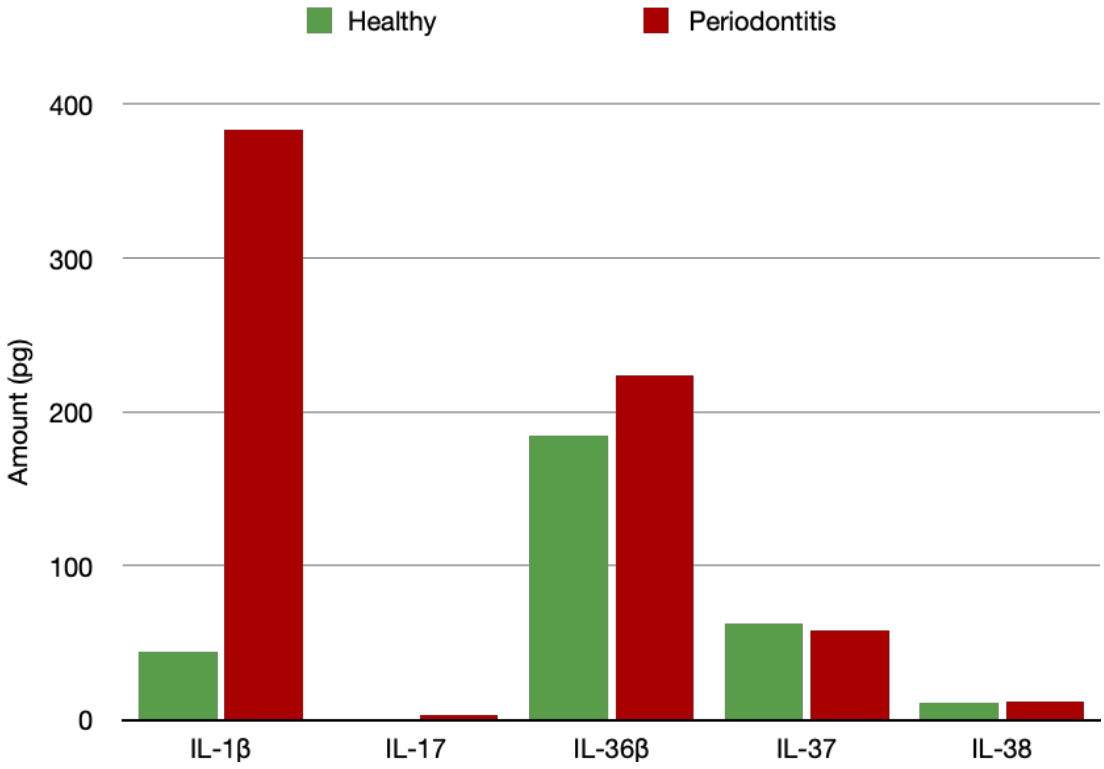


Table 9: GCF concentrations (pg/µl) of IL-1β, IL-17, IL-36β, IL-37, IL-38.

| Interleukin | | Healthy | Periodontitis | P-value |
|---|---|---|---|---|
| **IL-1β** | Mean (SD) | 37.69±49.49 | 183.70±173.29 | **<0.001**[*a] |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 18.51 (4.64-54.63) | 132.01 (73.30-244.47) | **<0.001**[*a] |
| **IL-17** | Mean (SD) | 0.455±0.62 | 1.34 ±0.89 | **<0.001**[*a] |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 0.23 (0.47-0.67) | 1.24 (1.24-2.05) | **0.002**[*b] |
| **IL-36β** | Mean (SD) | 182.86 ± 117.79 | 124.94±96.90 | **0.049**[*a] |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 158(93.80-244.18) | 89.8(58.87-159.86) | 0.088[b] |
| **IL-37** | Mean (SD) | 60.69±75.63 | 30.16±31.06 | **0.046**[*a] |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 37.44 (24.55-55.01 | 18.14 (10.58-46.18) | **0.017**[*b] |
| **IL-38** | Mean (SD) | 10.54 ±7.90 | 4.99±4.91 | **0.012**[*a] |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 8.66 (4.93-13.99) | 3.93 ( 0.43-8.60) | **0.011**[*b] |

* Statistically significant difference (P < .05).

a Determined by Mann-Whitney U test

b Adjusted for age and gender

Figure 6: Bar chart of concentrations (pg/ µl) of IL-1β, IL-17, IL-36β, IL-37, IL-38 in GCF of healthy and periodontitis subjects.

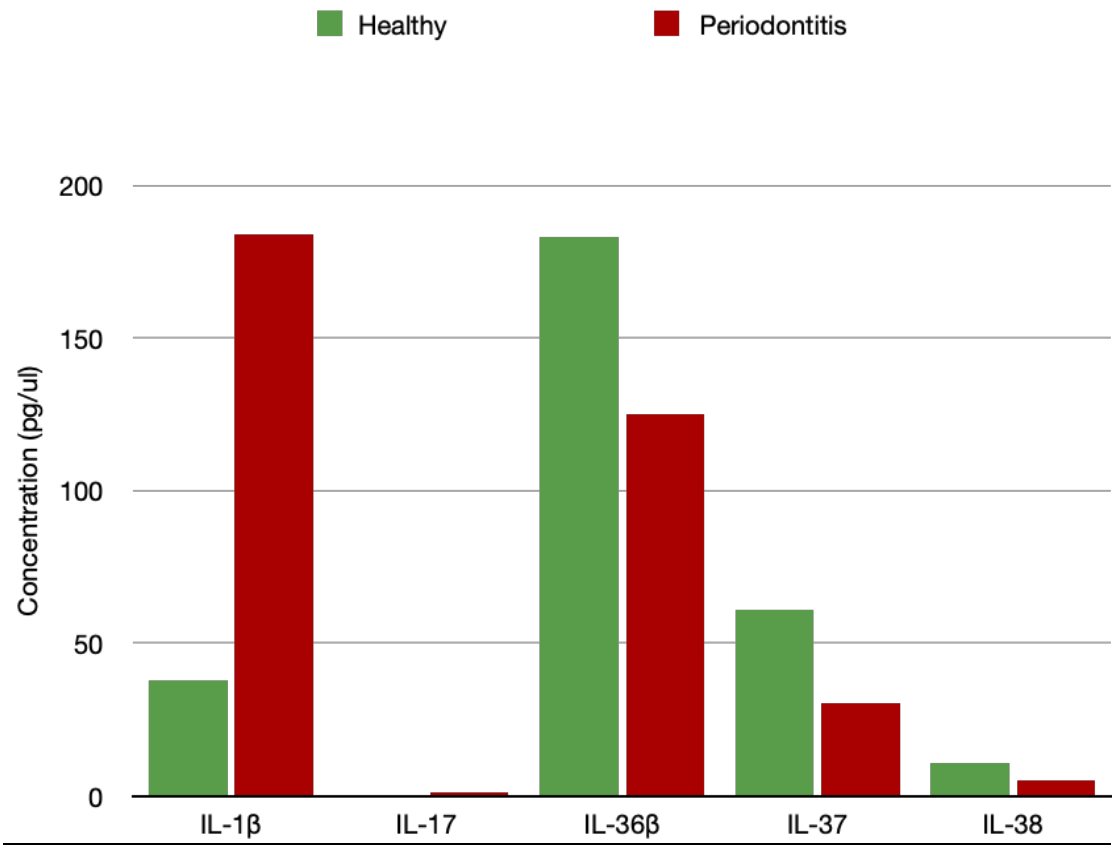
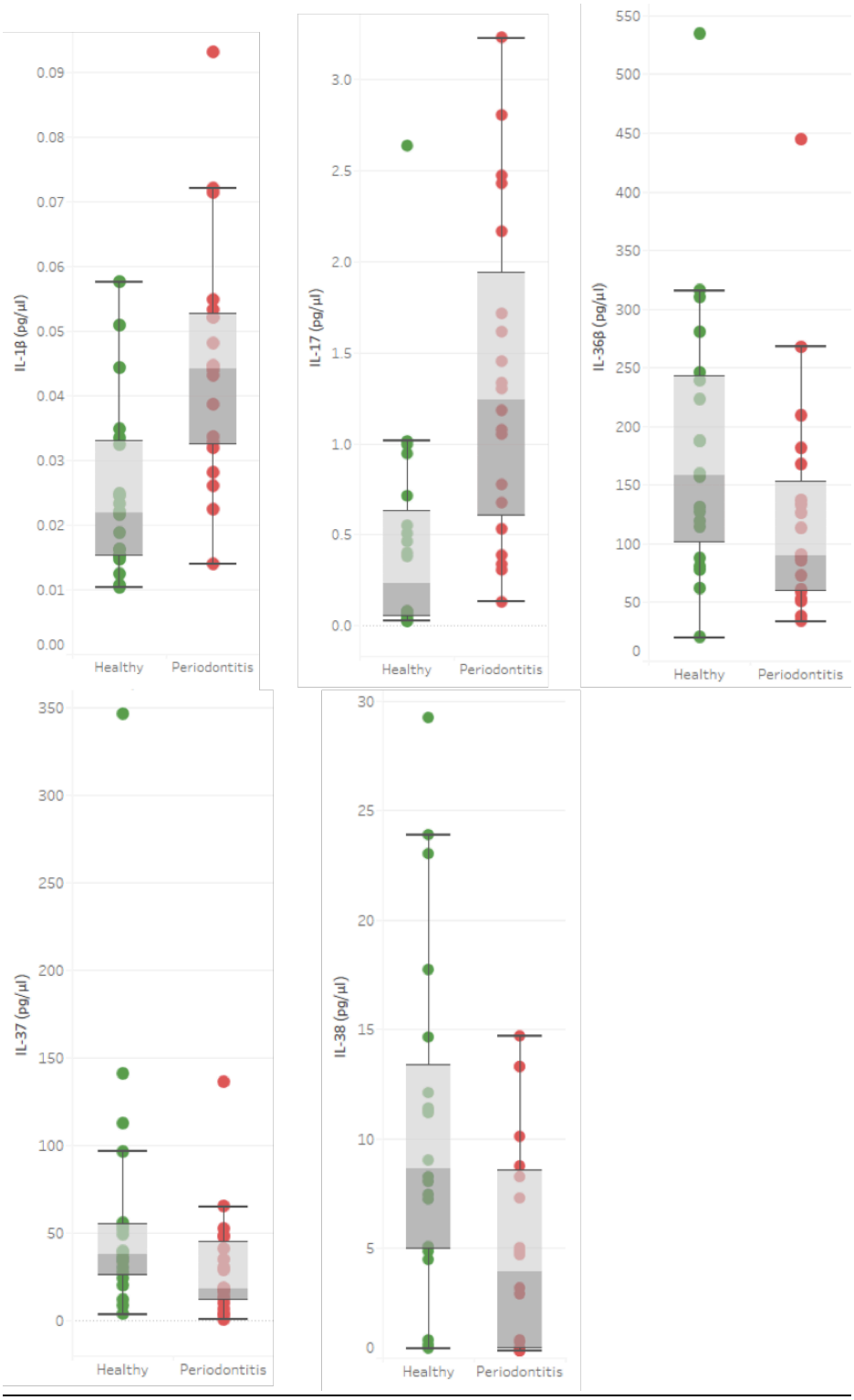


Figure 7: Box-plot of GCF concentrations (pg/ µl) of IL-1β, IL-17, IL-36β, IL-37, IL-38 in GCF of healthy and periodontitis subjects.



## 3.6 Pro-inflammatory and Anti-inflammatory index in GCF

The results of the pro-inflammatory and anti-inflammatory indices in GCF are shown in Table 10.

The pro-inflammatory and anti-inflammatory index ratios in GCF are depicted in Figure 7. There was a statistically significant difference in the pro-inflammatory and anti-inflammatory indices between healthy and periodontitis patients (p= 0.006).

The results suggest a significant increase in pro-inflammatory mediators which are IL-1$\beta$ IL-17 and IL-36$\beta$ and a significant decrease in anti-inflammatory mediators IL-37 and IL-38 in periodontitis patients compared to healthy subjects in GCF.

Figures 8 and 9 illustrate the pro-inflammatory and anti-inflammatory indices by group.

Table 10: GCF levels of Pro-inflammatory interleukins (IL-1β, IL-17, IL-36β) and Anti-inflammatory interleukins (IL-37, IL-38) in (pg/µl).

| Index | | Healthy (n=20) | Periodontitis (n=20) | P-value |
|---|---|---|---|---|
| **Pro-inflammatory (pg/µl)** | Mean ± SD | 5.53±4.80 | 20.23±23.55 | **0.006**[*a] |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 3.90 (2.14-8.20) | 11.16 (4.22-23.43) | |
| **Anti-inflammatory (pg/µl)** | Mean ± SD | 0.46±0.59 | 0.16±0.18 | **0.006**[*a] |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 0.25 (0.12-0.46) | 0.09 (0.04-0.23) | |

* Statistically significant difference (P < .05).

a Determined by Mann-Whitney U test

Figure 8: Pie-chart of pro-inflammatory index and anti-inflammatory index ratios in GCF of healthy and periodontitis subjects.

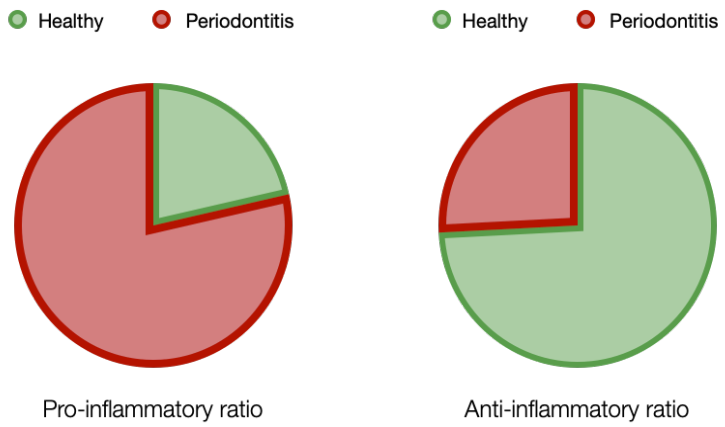
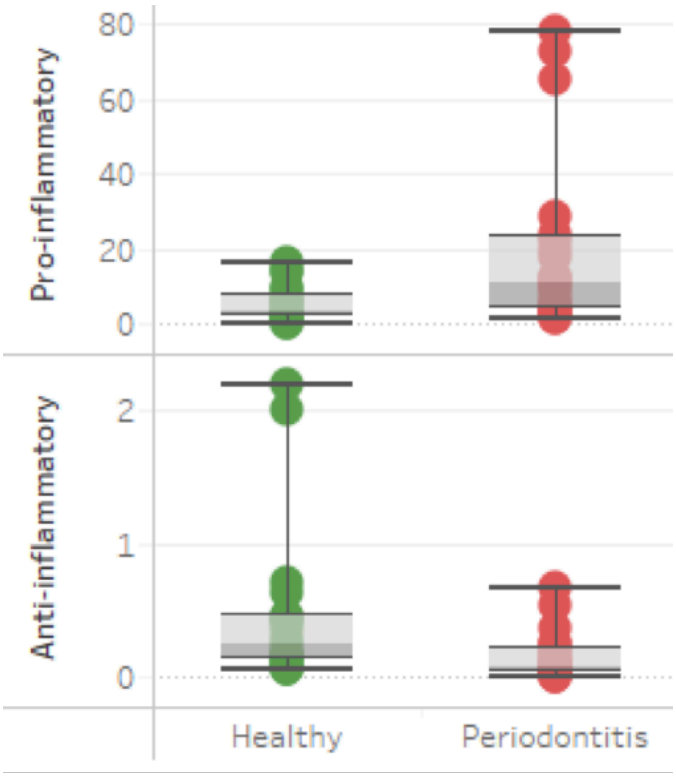



Figure 9: Box-plot of pro-inflammatory index and anti-inflammatory index ratios in GCF of healthy and periodontitis subjects.



## 3.7. Association between frequency of detection of interleukins in GCF with GCF volume and mean PD of sites of sample collection

Table 11 summarizes the results of the association analysis between frequency of detection of interleukins in GCF and GCF volume (µl) and mean PD of the site where GCF was collected.

Given that IL-1β and IL-36 had 100% frequency detection rates, no variability in the detection was found across the samples. The statistical analysis to explore relationships of the detection of IL-1β and IL-36 with volume and mean PD would not be informative.

For IL-17 our data showed a significant association with mean PD ($p < 0.001$), but a non-significant association with GCF volume.

The association between IL-37 and IL-38 levels with both volume and mean PD, were not statistically significant.

Table 11: Association between frequency of detection of interleukins in GCF with GCF volume (µl) and PD of site of sample collection.

| Interleukin[e] | | Detected | Not detected | P |
|---|---|---|---|---|
| IL-17 | N | 30 | 10 | 0.129[a] |
| | **Volume (µl)** | | | |
| | Mean (SD) | 1.74±0.67 | 1.37±0.64 | |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 1.80 (1.12-2.60) | 1.16 (0.80-1.10) | |
| | **Mean PD** | | | |
| | Mean (SD) | 6.00±6.50 | 5.85±0.24 | **<0.001**[*a] |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 6.50 (3.00-8.13) | 3.00 (2.50-3.00) | |
| IL-37 | N | 38 | 2 | 0.908[a] |
| | **Volume (µl)** | | | |
| | Mean (SD) | 1.70±0.68 | 1.64±0.84 | |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 1.64 (0.10-42.50) | 0.90 (1.04-2.70) | |
| | **Mean PD** | | | 0.864[a] |
| | Mean (SD) | 5.20±2.52 | 5.50±4.94 | |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 4.30 (3.00-7.12) | 5.00 (NA) | |
| IL-38 | N | 29 | 11 | 0.440[a] |
| | **Volume (µl)** | | | |
| | Mean (SD) | 1.60±0.72 | 1.80±0.58 | |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 1.67 (0.90-2.40) | 1.81 (1.20-2.23) | |
| | **Mean PD** | | | 0.104[a] |
| | Mean (SD) | 4.80±2.52 | 6.40±2.50 | |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 3.00 (3.00-7.00) | 7.00 (3.00-9.00) | |

* Statistically significant difference (P < .05).

a Determined by Mann-Whitney U test

e This test could not be conducted for IL-1β and IL-36β as they exhibited 100% detection.

NA The interquartile range could not be determined because the sample size is low.

## 3.8. Correlation of GCF amount of interleukins with GCF volume and mean PD of sites of sample collection

The correlation results of GCF amount of interleukins with GCF volume and with mean PD of sites of sample collection are shown in Table 12.

GCF amount of IL-1β and IL-17 showed a moderate positive correlation with GCF volume and a strong positive correlation with mean PD. Both correlations were statistically significant ($p < 0.001$).

GCF amount of IL-36β, IL-37 and IL-38 did not did not show any significant correlation with either GCF volume or mean PD of sites of sample collection.

Table 12: Spearman's correlation coefficient between GCF interleukins amount (pg) with GCF volume (µl) and mean PD of sites of sample collection.

| Interleukin | | Volume (µl) | Mean PD |
|---|---|---|---|
| **IL-1β** | rho | 0.559 | 0.747 |
| | p-value | **<0.001*** | **<0.001*** |
| **IL-17** | rho | 0.519 | 0.725 |
| | p-value | **<0.001*** | **<0.001*** |
| **IL-36** | rho | 0.065 | 0.189 |
| | p-value | 0.680 | 0.240 |
| **IL-37** | rho | 0.010 | -0.025 |
| | p-value | 0.950 | 0.917 |
| **IL-38** | rho | 0.182 | -0.066 |
| | p-value | 0.260 | 0.685 |

* Statistically significant difference ($P < .05$).

## 3.9 Correlation of GCF concentration of interleukins with GCF volume and mean PD of sites of sample collection

Table 13 shows the results of the correlational analysis between GCF interleukins concentration (pg/µl) with GCF volume (µl) and with mean PD of sites of sample collection.

GCF concentration of IL-1β showed a moderate positive correlation with GCF volume and with mean PD of sites of sample collection. Both correlations were statistically significant ($p < 0.001$).

GCF concentration of IL-17 showed a weak positive correlation with GCF volume that was not statistically significant but a moderate significant correlation with mean PD of sites of sample collection ($p<0.001$).

GCF concentration of IL-36β showed a statistically significant negative correlation with both GCF volume and mean PD of sites of sample collection ($p<0.001$, $p=0.030$ respectively).

GCF concentration of IL-37 and IL-38 showed a statistically significant negative correlation with both GCF volume and mean PD of sites of sample collection ($p<0.05$).

Table 13: Spearman's correlation coefficient between GCF interleukins concentration (pg/µl) with GCF volume (µl) and with mean PD of sites of sample collection.

| Interleukin | | Volume (µl) | Mean PD |
|---|---|---|---|
| **IL-1β** | rho | 0.388 | 0.658 |
| | p-value | **0.013*** | **<0.001*** |
| **IL-17** | rho | 0.196 | 0.576 |
| | p-value | 0.226 | **<0.001*** |
| **IL-36** | rho | -0.701 | -0.344 |
| | p-value | **<0.001*** | **0.03*** |
| **IL-37** | rho | -0.437 | -0.430 |
| | p-value | **0.005*** | **0.006*** |
| **IL-38** | rho | -0.322 | -0.376 |
| | p-value | **0.042*** | **0.017*** |

* Statistically significant difference (P < .05).

## 3.10 Correlation between GCF interleukins

Table 14 presents the results regarding the Spearman's correlation coefficients among GCF interleukins. It was observed that IL-17 showed a strong positive correlation with IL-1β, with a coefficient of 0.77 ($p < 0.001$). No other significant correlations were observed between IL-1β and any the other interleukins.

Table 14: Spearman's correlation coefficient between GCF interleukins.

| Interluekin | | IL-1B | IL-17 | IL-36B | IL-37 | IL-38 |
|---|---|---|---|---|---|---|
| **IL-1β** | rho | 1 | 0.77 | -0.096 | -0.265 | -0.264 |
| | p-value | - | **<0.001** | 0.555 | 0.098 | 0.099 |
| **IL-17** | rho | 0.77 | 1 | 0.097 | -0.335 | -0.26 |
| | p-value | **<0.001** | - | 0.552 | **0.035** | 0.105 |
| **IL-36β** | rho | -0.096 | 0.097 | 1 | 0.204 | 0.162 |
| | p-value | 0.555 | 0.552 | - | 0.207 | 0.317 |
| **IL-37** | rho | -0.265 | -0.335 | 0.204 | 1 | 0.261 |
| | p-value | 0.098 | **0.035** | 0.207 | - | 0.103 |
| **IL-38** | rho | -0.264 | -0.26 | 0.162 | 0.261 | 1 |
| | p-value | 0.099 | 0.105 | 0.317 | 0.103 | - |

* Statistically significant difference (P < .05).

## 3.11 Concentration of Interleukins in Saliva

The concentration was calculated by dividing the amount (pg) by saliva volume (ml). Table 15 presents the concentrations values of interleukins in saliva of the healthy and periodontitis groups as well as the p-values before and after adjustment for age, gender and saliva weight using the nonparametric Mann-Whitney U test and generalized linear model analysis accordingly.

IL-1β levels were significantly different between the groups, with periodontitis patients showing higher mean±SD salivary levels (45.70±70.13 pg/mL) compared to healthy individuals (17.91±28.2 pg/mL). This difference was significant after adjusting for age and gender (p= 0.029) and when adjusting for age, gender, and saliva weight (p= 0.005).

Due to low frequency of detection of IL-17 in saliva, only descriptive analysis was performed showing that the healthy group had mean±SD IL-17 concentration of 0.80±2.20

pg/mL, while the periodontitis group showed a mean±SD of 0.14±0.41 pg/mL. No comparisons between the healthy and periodontitis groups for salivary levels of IL-17 were conducted.

IL-36β also showed higher salivary concentration in periodontitis patients compared to healthy subjects, but the difference was not statistically significant even after adjustment for the saliva weight (p=0.410).

IL-37 salivary levels were higher in healthy subjects compared to periodontitis patients, but this difference was not statistically significant before and after the adjustments.

IL-38 salivary concentrations were higher in healthy subjects than in periodontitis patients without any significant difference before and after the adjustments.

The concentration of interleukins in saliva in the healthy and periodontitis groups is also illustrated in Figure 10.

Table 15: Salivary concentrations (pg/ml) of IL-1β, IL-17, IL-36β, IL-37, IL-38.

| Interleukin | | Healthy | Periodontitis | P-value |
|---|---|---|---|---|
| **IL-1β** | Mean (SD) | 17.91±28.2 | 45.70±70.13 | 0.052[a]<br>**0.0290**[*b]<br>**0.005**[*c] |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 2.20 (0.60-26.04) | 6.90 (2.92-81.6) | |
| **IL-17** | Mean (SD) | 0.80±2.20 | 0.14±0.41 | |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 0 | 0 | |
| **IL-36β** | Mean (SD) | 653.4±466.8 | 691.40±455.04 | 0.620[a]<br>0.530[b]<br>0.410[c] |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 612.6 (277.50-989.40) | 594.8 (352.83-838.32) | |
| **IL-37** | Mean (SD) | 44.61±55.8 | 28.41±40.06 | 0.607[a]<br>0.418[b]<br>0.412[c] |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 21.94 (3.26-75.1) | 10.2 (4.32-43.91) | |
| **IL-38** | Mean (SD) | 41.20±48.30 | 24.61±39.30 | 0.106[a]<br>0.139[b]<br>0.092[c] |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 24.3 (0.77-70.91) | 5.90 (0.38-38.91) | |

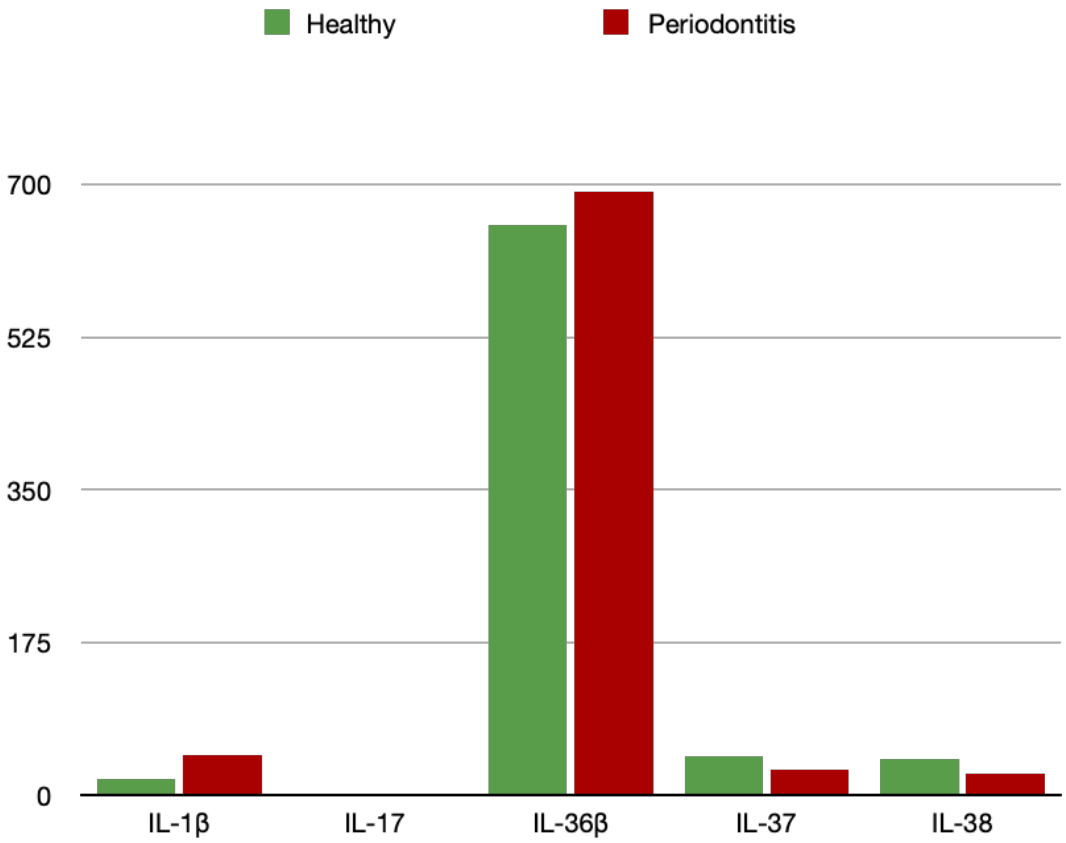
* Statistically significant difference (P < .05).

a Determined by Mann-Whitney U test.

b Adjusted for age and gender.

c Adjusted for age and gender and saliva weight.

Figure 10: Bar chart of concentrations (pg/ml) of IL-1β, IL-17, IL-36β, IL-37, and IL-38 in the saliva of periodontitis patients and healthy subjects.




## 3.12. Pro-inflammatory and Anti-inflammatory index in saliva

Table 16 shows the results of the pro-inflammatory and anti-inflammatory index in saliva that were calculated in the same manner as described earlier in GCF (3.7). The mean salivary levels of pro-inflammatory interleukins were higher in periodontitis patients compared to healthy subjects, but the difference was not statistically significant (p=0.068).

Salivary levels of anti-inflammatory interleukins were higher in healthy subjects compared to the periodontitis patients but not statistically significant (p=0.068). Figure 11 presents pie-charts depicting the results, and Figure 12 shows box-plots of the indexes.

Table 16: Salivary Concentrations of Pro-inflammatory interleukins (IL-1β, IL-17, IL-36β) and Anti-inflammatory interleukins (IL-37, IL-38).

| Index | | Healthy (n=20) | Periodontitis (n=20) | P-value |
|---|---|---|---|---|
| **Pro-inflammatory** | Mean ± SD | 38.2±98.12 | 100.5±303.11 | 0.068[a] |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 9.10 (4.60-16.6) | 26.53 (5.93-48.61) | |
| **Anti-inflammatory** | Mean ± SD | 0.27±0.55 | 0.103±0.144 | 0.068[a] |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 0.10 (0.06-0.22) | 0.037 (0.02-0.16) | |

* Statistically significant difference (P < .05).

a Determined by Mann-Whitney U test.

Figure 11: Pie-charts of Pro-inflammatory index and Anti-inflammatory index ratios in the saliva of healthy and periodontitis subjects.

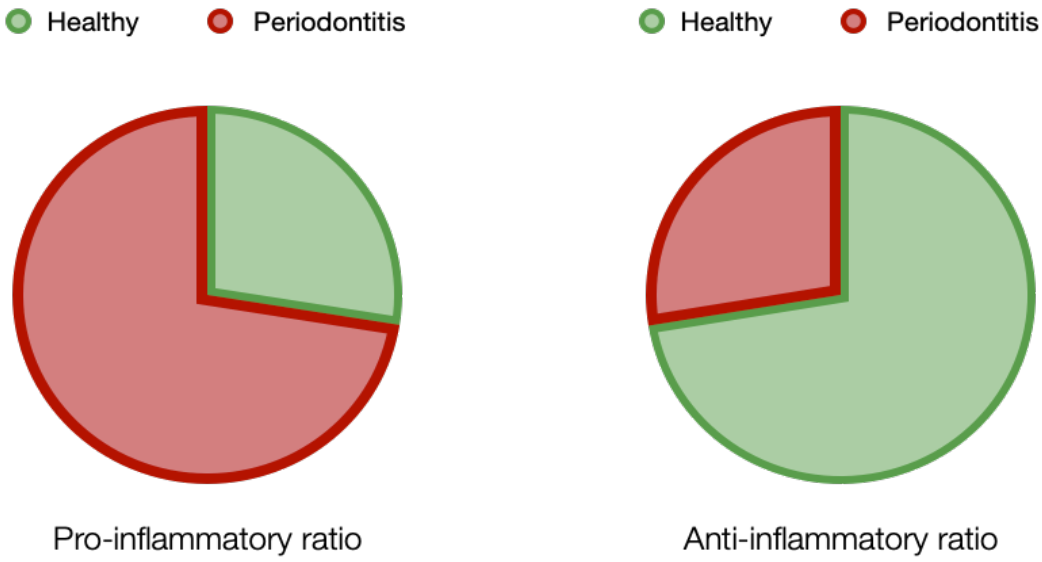
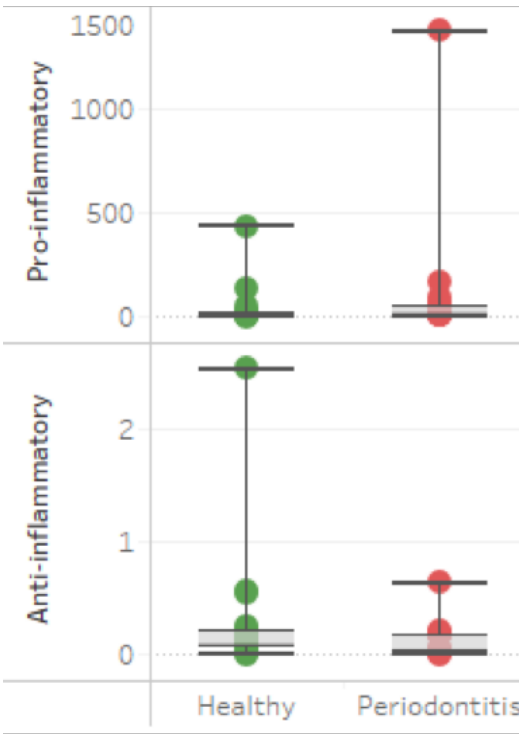


Figure 12: Box-plots of Pro-inflammatory index and Anti-inflammatory Index ratios in the saliva of healthy and periodontitis subjects.




## 3.13. Association between Salivary weight and Frequency of detection of Interleukins

Table 17 presents the results of the association analysis between calculated salivary weight and the detection frequency of the investigated interleukins.

The association between saliva weight and IL-1β and IL-36β was not calculated since both interleukins were detected in 100% of salivary samples, leaving no variability in detection frequency to correlate with saliva weight.

There was a statistically significant association between saliva weight and IL-17 (p= 0.018). All other interleukins did not show any significant correlations with saliva weight.

Table 17: Association between calculated saliva weight and detection frequency.

| Interleukin[e] | Calculated saliva weight | Detected | Not detected | P |
|---|---|---|---|---|
| **IL-17** | N | 8 | 32 | **0.018**[*a] |
| | Mean (SD) | 3.04±1.20 | 5.90±3.23 | |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 3.01 (2.03-4.30) | 4.30 (3.60-8.12) | |
| **IL-37** | N | 38 | 2 | 0.974[a] |
| | Mean (SD) | 5.01±3.05 | 6.30±5.29 | |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 4.06 (3.50-5.01) | 6.30 (NA) | |
| **IL-38** | N | 25 | 15 | 0.639[a] |
| | Mean (SD) | 4.98±3.22 | 5.23±3.01 | |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 10.37 (3.27-6.40) | 4.50 (3.40-5.21) | |

* Statistically significant difference (P < .05).

a Determined by Mann-Whitney U test

e This test could not be conducted for IL-1β and IL-36β as they exhibited 100% detection.

NA The interquartile range could not be determined because the sample size is low.

## 3.14 Correlation between Salivary Levels of Interleukins and Salivary Weight

Table 18 focuses on the correlation between salivary weight and salivary concentration of interleukins.

There was a significant negative correlation between IL-17 concentration and the weight of saliva (rho=-0.348; p=0.028). The correlations between IL-1β, IL-36β, IL-37, and IL-38 and salivary weight were not statistically significant.

Table 18: Spearman's correlation coefficient between salivary levels of interleukins (pg/ml) and salivary weight.

| | Interleukins | IL-1β | IL-17 | IL-36β | IL-37 | IL-38 |
|---|---|---|---|---|---|---|
| **Calculated saliva weight** | rho | -0.251 | -0.348 | -0.250 | -0.095 | 0.138 |
| | p-value | 0.118 | **0.028*** | 0.120 | 0.562 | 0.394 |

* Statistically significant difference (P < .05).

## 3.15 Correlation between Saliva and GCF cytokines concentrations

Table 19 presents the Spearman's correlation coefficients and the p-values for the relationship between cytokines' concentrations in saliva and GCF.

IL-1β in saliva showed a strong and statistically significant correlation with IL-1β in GCF with a correlation coefficient of 0.574 and a p-value <0.001. Similarly, IL-38 demonstrated a significant positive correlation, with a coefficient of 0.536 and a p-value <0.001. In contrast, IL-17 and IL-36β did not show significant correlations between saliva and GCF, with coefficients of 0.067 and 0.051 and p-values of 0.681 and 0.754 respectively. IL-37 presented a slight negative correlation between saliva and GCF; however, the correlation was not statistically significant,

with a coefficient of -0.037, and a p-value of 0.821. These results suggest a variable association between specific inflammatory mediators in saliva and GCF.

Table 19: Spearman's correlation coefficient between calculated interleukins concentration levels in Saliva (pg/ml) and GCF (pg/µl).

| Saliva mediators | GCF mediators | Correlation Coefficient | P-value |
|---|---|---|---|
| **IL-1β** | IL-1B | 0.574 | **<0.001*** |
| **IL-17** | IL-17 | 0.067 | 0.681 |
| **IL-36β** | IL36b | 0.051 | 0.754 |
| **IL-37** | IL-37 | -0.037 | 0.821 |
| **IL-38** | IL-38 | 0.536 | **<0.001*** |

* Statistically significant difference (P < .05).

## 4. DISCUSSION

This cross-sectional study aimed to evaluate the differences in salivary and GCF levels (amount and concentration) of interleukins IL-1β, IL-17, IL-36β, IL-37, and IL-38 between periodontitis patients and healthy subjects. Our study showed significant differences in the level of the investigated interleukins between periodontitis patients and healthy subjects.

The specific selection criteria method used in our study permitted a clear distinction between healthy and periodontitis subjects, allowing us to compare the levels of the cytokines. Periodontitis patients presented significantly higher PD, CAL, PI, BOP, suppuration and mobility than healthy subjects ($p < 0.001$). We also followed very specific criteria for GCF sample site collection for all subjects. All GCF collection sites in periodontitis patients had PD $\geq$5 mm (mean 7.57$\pm$1.44 mm), CAL >3 mm (mean 7.90$\pm$2.19 mm), and 100% BOP, while all healthy subjects had PD & CAL < 3mm and absence of BOP.

We used the intracrevicular approach for GCF sampling with perio paper strips in all subjects keeping the periopaper in the sulcus for exactly 30 seconds or before 30 seconds when the periopaper was visibly wet as a standardized method. Different GCF sampling methods have been previously reported in the literature other than the technique followed in our study [47] [48]. One frequently described method is the intracrevicular washing technique, which includes an ejection needle and a collection needle; this technique described by Salonen and Puanio in 1991[49] is not common due to increased technique sensitivity and high possibility of contamination of GCF fluid with blood. Another method is the microcapillary technique using a calibrated pipette with an a priori-determined set volume for GCF collection. The drawback of this technique is the duration of the sample collection. Previous studies have reported an

extended collection duration, especially in healthy sites that patients can not tolerate. Some studies have allocated 10 minutes as the maximum time for collection, which could also increase the possibility of contamination[50]. In our study, the intracrevicular approach with perio paper strips was followed in all subjects, keeping the periopaper in the sulcus for exactly 30 seconds or before 30 seconds when the periopaper was visibly wet as a standardized method.

We then quantified GCF volume absorbed in perio paper strips using Periotron 8000. We frequently calibrated the device (almost once a month) using distilled water that has similar viscosity to GCF in order to achieve higher estimate of the volume of GCF. We used supplemented software to convert the volume of the samples to ul referring to a standardized curve [51]. GCF volume was found to be higher in sites collected from periodontitis patients compared to healthy subjects in our study. This finding aligns with previous studies that suggested that the increased vascular permeability in the inflammation site of periodontal tissues of periodontitis patients increases GCF volume [52].

Sensitive techniques are required to accurately measure proteins and inflammatory mediators in biological samples of low volumes such as GCF. The most widely used quantification analysis for cytokines is enzyme-linked immunosorbent assay (ELISA). Although ELISA is a sensitive analysis that has been frequently used it allows only a small panel of cytokines (usually one or two) to be analyzed, limiting the ability to detect multiple cytokines in a low volume collected such as GCF. On the other hand, Multiplex Luminex analysis technology was selected in our study as it offers the advantage of allowing simultaneous detection and quantification of multiple cytokines within a small-volume sample [53].

Both IL-1β and IL-36β were detected in 100% of GCF samples from both periodontitis patients and healthy subjects, confirming that these interleukins are consistently present in

periodontal tissues regardless of the state of periodontal health. IL-17 was detected in 100% of the periodontitis group's samples and only 50% of the healthy group's samples, a difference that was statistically significant ($p < 0.001$). IL-37 was detected in 95% of samples from both groups, showing no significant difference in frequency of detection between periodontitis and healthy subjects. Lastly, IL-38 was present in 85% of healthy subjects' samples and 60% of periodontitis patients' samples; however, this difference was not statistically significant.

The problem of missing values in proteomic research is very common but rarely reported on how it was handled by investigators raising questions about the reproducibility of research experiments and the validity of data . Cytokines can often be found below the detection limit due to technical errors, use of antibodies with reduced sensitivity or real absence in biological samples.[54] Different methods of replacing missing values have been discussed to translate the data and the proteomics technologies to valuable biological and appropriate data [55-57]. We used here the data imputation method as it ensures the usability of data using small values from that fall within the range of the dataset[45,46]. The samples (IL-17, IL-37, and IL-38) that had values below the Luminex detection threshold were given a value by dividing the detection limit of each cytokine by five (detection limit/5). This approach is known as a limit of detection (LOD)-based imputation method [44,45].

**4.1 Pro-inflammatory interleukins in GCF**

Our findings showed higher levels of pro-inflammatory cytokines (IL-1β, IL-17) in periodontitis patients compared to healthy subjects in GCF samples, confirming results from previous studies.[20,23] Our data enhances our understanding that periodontitis is an exacerbated

inflammatory response to oral bacterial plaque and interleukins play an important role in the process of bone resorption and tissue destruction [20] [21].

Correlational findings (Table 12) showed the relationship between the levels of the cytokines investigated and PD of GCF sample site collection. Both IL-1β and IL-17 in GCF showed a positive significant correlation with the mean PD and volume of GCF sample site collection. Furthermore, we also show a strong positive correlation between IL-1β and IL-17 (Table 12) indicating that higher levels of IL-1β are associated with higher IL-17 levels in humans, highlighting their role in periodontitis and suggesting their possible synergistic effect in periodontitis severity.

We observed a significantly higher concentration of IL-36β in GCF of healthy subjects (182.86±117.79 pg/ul) compared to periodontitis patients (124.94±96.90 pg/ul) (p=0.049). This finding is consistent with the study by Kursunlu et al, who reported that the mean ± SD amount of IL-36β in GCF was 7.86±2.08 pg in aggressive periodontitis patients, 7.01 ± 4.75 pg in chronic periodontitis patients, and 6.28±1.71 pg in healthy subjects. Additionally, the concentrations were 35.84±28.60 pg/ul for aggressive periodontitis, 35.16±25.21 pg/ul for chronic periodontitis, and significantly higher at 78.15±48.22 pg/ul in healthy individuals [27].

As a member of the IL-1 family, a possible dual role of IL-36 in periodontitis pathogenesis promoting inflammatory responses that exacerbate tissue destruction but also contributing to the resolution phase of inflammation aiding tissue repair and healing [6]. However, the scientific evidence for IL-36β is still limited and more studies are needed to understand better the role of IL-36β in the pathogenesis of periodontitis.

### 4.2 Anti-inflammatory interleukins in GCF

IL-37 plays a protective role against periodontal inflammation. IL-37 has been considered to inhibit the production of pro-inflammatory cytokines, suggesting its function in suppressing inflammatory responses [6,58].

In our study, the concentration of IL-37 was significantly higher in GCF of healthy subjects (60.69±75.63 pg/ul) compared to periodontitis patients (30.16±31.06 pg/ul) (P=0.046) (without any adjustments) and after adjusting for age and gender (P=0.017). Similar findings were reported in a previous study by Saglam et al. in 2015, in which the GCF concentration of IL-37 was lower in chronic periodontitis group compared to healthy group [30]. In contrast, a study by Ho et al. reported a higher GCF level of IL-37 in a periodontitis group compared to healthy subjects [59]. The different results could be attributed to the methodology of GCF collection; the latter study collected GCF from 6 different sites in each subject and were pooled together for analysis using periopaper strips in the gingival crevice for 60 seconds, while in both our study and Saglam et al, paper strips were left in place for 30 seconds, collected, and pooled from two different sites and analyzed separately. [47]

Our findings about low levels of IL-37 and high levels of IL-1β in the periodontitis subjects are very interesting and align with previous genome wide association study (GWAS) findings [29], highlighting the downgrade of IL-37 in the upregulation of IL-1β and the promotion of a hyper-inflammatory periodontal environment [29]. In this GWAS study IL-37 was only studied at the mRNA expression level [29]. However, our study shows first to the best of our knowledge that downregulated expression of IL-37 may upregulate IL-1β expression at the proteomic level. Further studies are suggested to dissect potential connection mechanisms at the molecular level

and clinically evaluate how IL-1β/IL-37 ratio could be used as a potential diagnostic and therapeutic biomarker for periodontitis.

IL-38 has been previously reported to be involved in Th-17-dependent inflammatory autoimmune diseases, with most studies concluding its anti-inflammatory effect in RA, psoriasis, SLE, and IBD [60]. Its role in periodontitis has not been widely investigated. Our study found that IL-38 concentration was significantly higher in healthy subjects (10.54±7.90 pg/ul) compared to those with periodontitis (4.99±4.91 pg/ul) ($p=0.012$). In contrast to our findings, Korkmaz et al. reported higher GCF levels of IL-38 in patients with periodontitis (8.18±2.95 pg/ul) compared to healthy subjects (4.27±0.82 pg/ul) ($P<0.001$) [34]. However, the levels of IL-38 were reported in amount (pg/30s) without making any references to the volume of GCF, which is important to normalize data of amount of cytokine into concentration. While Korkmaz et al. reported the total amount of cytokines, our study also reported the concentration levels that are more representative of the interleukin profile in the sample. In addition, Korkmaz et al. found a significant positive correlation between IL-38 amount with PD of GCF sample site collection. However, in our study the correlation between IL-38 amount with mean PD of GCF sample site collection was non-significant (Table 12).

Furthermore, our findings show that IL-38 concentration levels are inversely related to periodontal disease severity, supporting the anti-inflammatory role of IL-38. The correlational analysis showed a statistically significant negative correlation with GCF volume and the mean PD of GCF sample site (Table 13).

**Saliva**

Whole unstimulated saliva was collected to explore the levels of inflammatory mediators in the whole oral cavity of patients. Saliva contains nonsalivary elements such as GCF, immune cells, and tissue metabolites [61]. Unstimulated saliva is mostly chosen compared to stimulated saliva to avoid any dilution in the concentration of biomarkers [62].

The frequency of detection patterns for interleukins in saliva samples of our patients were 100% for IL-1β and IL-36β. However, IL-17 showed a low detection rate of only 20% in both the periodontitis and healthy groups. IL-37 was consistently detected in 95% of samples from both groups. IL-38 was found in 50% of periodontitis patients' samples and 75% of healthy saliva samples, without any statistically significant difference in the frequency of detection between the groups.

**4.3 Pro-inflammatory interleukins in saliva (IL-1β, IL-17, IL-36β)**

Salivary biomarkers have been proposed to predict periodontal disease status and progression as saliva is a noninvasive diagnostic fluid without showing any sensitive technique of collection [63] [64]. IL-1β is the most researched saliva biomarker for periodontitis. A recent systematic review and meta-analysis by Arias-Bujanda et al. indicated that IL-1β had the highest value of sensitivity for periodontal diagnosis, reaching up to 78.7% and a specificity of 78% [17]. A more recent investigation showed that periodontitis patients with progressed sites exhibited elevated levels of salivary inflammatory biomarkers, including IL-1β, and experienced a significant decrease the inflammatory biomarkers after non-surgical periodontal therapy [64]. In the present study, salivary levels of IL-1β were significantly higher in periodontitis patients,

consistent with our findings from GCF levels of IL-1β. This aligns with the predominant role of IL-1β in leading the inflammatory processes in the oral cavity.

Since frequency of detection of IL-17 was 20% in both studied groups, the analysis of IL-17 was strictly descriptive without proceeding to any comparisons between studied groups. The mean ± SD concentration of four samples detecting IL-17 in healthy subjects was (0.8±2.2 pg/ml) compared to four samples detecting IL-17 in periodontitis patients (0.14±0.41 pg/ml). Another study reported a lower salivary concentration of IL-17 in a Turkish population with periodontitis (3.15±9.35 pg/ml) compared to the healthy group (4.57±10.22 pg/ml). However, the difference was not statistically significant [24]. A contradictory study by Yang et al. reported that the IL-17 concentration was significantly higher in periodontitis patients (7.74±1.89 pg/ml) than in healthy subjects (3.39±1.20 pg/ml). However, both studies failed to report the detection frequency of IL-17 in saliva samples following a similar absence to report in several similar studies. Isaza-Guzman et al. reported that IL-17 was detected in 8.7% of Colombian population saliva samples, and no relationship between diagnosis of periodontitis and salivary IL-17 levels was found [65]. The varying results of IL-17 in saliva could be due to different sample sizes, definitions of diseases and inclusion criteria, amount of saliva collected, the collection of stimulated or unstimulated saliva, and time of collection during the day. More studies are needed to investigate why IL-17 is detected in a smaller percentage of salivary samples compared to GCF samples although the same antibodies from Multiplex immunoassay analysis were used for both saliva and GCF analysis in our study.

IL-36β levels in saliva were higher in periodontitis patients (691.4±455.01 pg/ml) compared to healthy subjects (653.4±55.8 pg/ml), an opposite finding compared to the GCF levels of IL-36β from our study; however, the difference did not show statistical significance.

The role of salivary IL-36β in periodontitis is yet to be determined. IL-36β has been known to have a pro-inflammatory role in autoimmune diseases. Serum levels of IL-36β were found to be upregulated in rheumatoid arthritis patients [26,66]. A study concluded that IL-36 family members (IL-36α, IL-36β, and IL-36γ) play a role in inflammatory bowel disease, with a significantly higher expression in patients with active disease compared to control subjects and found different patterns of expressions in Ulcerative colitis (UC) and Crohn's disease (CD). Furthermore, IL-36β was found to be higher in active CD compared to active UC and controlled UC, suggesting IL-36β as a diagnostic tool for inflammatory bowel disease [67]. Serum levels of IL-36β and IL-36γ were significantly higher in patients with another inflammatory disorder, Neuromyelitis Optica spectrum disorder, compared to healthy subjects. Interestingly, the levels of IL-36β and IL-36γ were observed to decrease in disease remission [68]. No other studies have investigated the salivary levels of IL-36β, limiting our ability to compare the results. Our study seems to be to the best of our knowledge the first study to report IL-36β in saliva, and thus, further studies are needed to explore the role of IL-36β in periodontal disease.

**4.4 Anti-inflammatory interleukins in saliva (IL-37, IL-38)**

In our study, salivary IL-37 and IL-38 exhibited higher levels in healthy subjects compared to patients with periodontitis; however, this difference was not statistically significant. The protective, regulating role of these cytokines in healthy individuals may limit the inflammatory response and delay the development of tissue-destructive processes that are typical of periodontitis. Contrary to our findings, Saglam et al. reported that the salivary concentrations of IL-37 were similar in all study groups (healthy, gingivitis, and periodontitis) [30]. Similar to our results, a recently published cross-sectional study evaluated salivary IL-38 levels and found that

IL-38 levels were higher in healthy subjects compared to periodontitis and gingivitis patients [69]. Korkmaz et al. reported higher levels of salivary IL-38 in periodontitis patients compared to heathy subjects, contradicting our findings [34].

The correlational analysis between saliva and GCF interleukins showed a significant correlation of IL-1β and IL-38 in both saliva and GCF, indicating that they might have a parallel role in the inflammatory process in the oral environment. Cytokines have a "yin-yang" role in regulating the host immune response which can explain the increased expression of IL-38 in response to IL-1β to counteract the deleterious pro-inflammatory effects of IL-1β. On the other hand, Toraman et al. detected a negative correlation between salivary IL-38 and IL-1β levels, but it was not statistically significant [69]. The lack of significance shown in the link between IL-17, IL-36β, and IL-37 highlights the complex nature of cytokine modulation and the potential independent pathways impacting their concentrations in both saliva and GCF. Further research might explore the mechanisms driving these differences, potentially offering further insights into their distinct roles.

The dynamic relationship between pro-inflammatory and anti-inflammatory cytokines is critical in understanding how the host regulates the immune system. Pro-inflammatory cytokines contribute to initiating and sustaining the inflammation, while anti-inflammatory cytokines help in the regression of the inflammation promoting its resolution and the return to tissue homeostasis. The balance between these two arms is needed to achieve the desired pathogen elimination without excessive tissue damage [6,70]. An imbalance in the "yin-yang" dynamics of these cytokines could lead to either a hyper-inflammatory response in the case of increased pro-inflammatory cytokines or an impaired immune system unable to battle pathogens properly in the case of reduced anti-inflammatory cytokines [70,71].

Our study highlights a significant elevation of pro-inflammatory index investigated (IL-1β, IL-17 and IL-36β) in GCF samples of subjects with periodontitis (20.23±23.55 pg/ul) compared to healthy subjects (5.53±4.80 pg/ul) (P=0.006). However, the difference between pro-inflammatory and anti-inflammatory indices in saliva samples did not reach statistical significance (P = 0.068).

Additional research is necessary to study the interaction of interleukins in periodontitis, understand how they influence each other, and fully comprehend the disease's pathogenesis. The application of personalized medicine in periodontal clinics can help identify cases that are at high risk of progressing into severe conditions. By deeply understanding the mechanism of the disease, a more targeted approach can be implicated and avoid missing the opportunity for early prevention and treatment [37].

FDA-approved therapeutic drugs (Anakinra, Rilonacept, and Canakinumab) have been developed targeting the pro-inflammatory activity of IL-1β and IL-1α in inflammatory diseases such as rheumatoid arthritis [6,72]. Recombinant IL-37 has shown protective effects in different inflammatory models, while IL-38 is increasingly being investigated for its therapeutic use in modulating inflammation [6,58,73,74]. Another drug being investigated is an NLRP3 inflammasome (Nod-like receptor protein 3) inhibitor. NLRP3 inflammasome pathway activation leads to the secretion of inflammatory cytokines, and its inhibition has shown promise in treating inflammatory disease [75]. Furthermore, recent studies have been focusing on the therapeutic use of Specialized Proresolving Mediators (SPMs) that promote the termination of intlammation such as Resolvins and Lipoxins[76]. These mediators have been found to restrict the inflammation process and promote the resolution of inflammation and return to tissue homeostasis [77,78].

In addition to the high cost, targeting specific cytokines can lead to significant side effects, such as an increased risk of cancer and infections due to diminished host defenses, as well as the potential development of progressive central nervous system disorders. Consequently, their use has been limited [79]. Due to these concerns, such treatments have not yet been widely adopted for periodontitis and need to be further explored.

### 4.5 Strengths and Limitations of this study

The strengths of our study include the meticulous selection criteria for enrolling patients, allowing clear differentiation between the two groups as well as the specific criteria for GCF sample site collection. Another strength is our decision to use the Luminex technology to be able to simultaneously investigate different mediators within the expected low GCF volume, which cannot be achieved using other techniques like ELISA. Furthermore, we reported the detection frequency, amount and concentration of each cytoking, while many other similar studies failed to report.. Moreover, we applied adjusted analysis for cofounders (age, gender) in a small sample size (N=20 per group).

Our study's cross-sectional design did not allow any prospective insights into the levels of interleukins in GCF and saliva or the effects of periodontal treatments on interleukin concentrations and progression of periodontal disease. Longitudinal studies are needed to fulfil this goal. Another limitation is the sample size which was determined after referring to previous studies on GCF levels of IL-37 and IL-36β, and not saliva. This might explain the low detection percentage of salivary IL-17. An additional limitation is the absence of assessing bacterial plaque at sites of GCF collection or overall in the mouth [80]. The generalizability of our findings is limited due to the convenience of the sampling method used to recruit subjects. Furthermore,

relying on self-reported medical histories might lead to the inclusion of subjects with certain undiagnosed systemic conditions, including Sjögren's disease, which could explain the low volume of saliva collected in a small number of enrolled subjects. These limitations might have had an impact on the overall interleukins levels and the lack of signfinicance between the two groups, especially in saliva.

Future studies should investigate interleukin levels before and after periodontal intervention. Using our novel data on salivary levels of several inflammatory mediatiors will now allow us to determine better and recalculate the power and sample size needed for a similar study, potentially yielding more significant results. Accurate assessment of the subject's health status by obtaining the medical history from their health care providers' medical records is needed.

Despite these limitations, our study contributes to a deeper understanding of the pathogenesis of periodontitis, confirming the critical role of specific interleukins in its pathogenesis. The significant elevation of IL-1β and IL-17 in GCF from periodontitis patients supports their role in the disease mechanism and highlights their potential utility as biomarkers for disease severity. Thus far, GCF IL-38 levels have only been examined in one study[34]. The results of our study show that healthy subjects have higher levels of IL-38 in GCF compared to periodontitis patients. This finding is important since it confirms the possible anti-inflammatory role of IL-38 in periodontal tissues. Our findings support the established anti-inflammatory roles of IL-37 and IL-38 in autoimmune disorders and also point to a possible protective role in preserving periodontal health highlighting their importance to be therapeutic targets in periodontal therapy. We believe that our study lays the groundwork for future investigations that can explore the profile of these interleukins and their correlation and ratios with other inflammatory mediators

before and after periodontal therapy as well as during periodontal maintenance and disease progression.

The clinical translation of our study pave the way of a chairside diagnostic test development incorporating these cytokines, along with other mediators. This would allow for a precise early diagnosis, monitoring and therapeutic outcome evaluation leading to a better, more centered patient treatment and disease management.

Second clinical translation of our findings is in therapeutic innovation. There is potential for the development of drugs that aid in modulating the activity of the cytokines, monoclonal antibodies based drugs that not only block the pro-inflammatory cytokine activity but also promotes the anti-inflammatry cytokine activity and production such as IL-37 and IL-38. Moreover, developing vaccines that upregulate the anti-inflammatory cytokines. The development of mRNA vaccines and the focus on personalized vaccine have been increasingly investigated as a form of immunotherapy in various cancer treatments [81]. More studies are essential for development of therapeutic technologies that provide a safe and an effective diagnostic and treatment modality in clinical settings.

# 5. CONCLUSIONS

Our study supports our hypothesis that periodontitis patients have different IL-1β, IL-17, IL-36β, IL-37 and IL-38 GCF and salivary profiles compared to periodontally healthy subjects.

1. IL-1β and IL-17 levels (amount and concentration) are significantly higher in GCF of periodontitis patients.
2. IL-36β concentration in GCF is lower in periodontitis patients, but this significance is lost after adjustment for age and gender.
3. IL-37 and IL-38 concentration levels in GCF are significantly lower in periodontitis patients.
4. Pro-inflammatory index in GCF is significantly higher in periodontitis patients.
5. Anti-inflammatory is in GCF is significantly higher in healthy subjects.
6. IL-17 detection frequency and concentration in GCF positively correlate with mean PD.
7. IL-1β and IL-17 amounts strongly correlate with mean PD and GCF volume.
8. IL-1β concentration in GCF moderately correlates with mean PD and GCF volume.
9. IL-36β concentration in GCF negatively correlates with mean PD and GCF volume.
10. IL-37 and IL-38 concentrations in GCF negatively correlate with mean PD and GCF volume.
11. IL-17 concentration in GCF strongly correlates with IL-1β.
12. Lower IL-37 levels may lead to increased IL-1β levels in GCF at the proteomic level.
13. IL-1β concentration in saliva is significantly higher in periodontitis patients.
14. No significant differences in salivary pro-inflammatory and anti-inflammatory indices was found between study groups.
15. IL-17 detection and concentration in saliva negatively correlate with saliva weight.

16. Salivary IL-1β concentration strongly correlates with GCF IL-1β concentration.

17. Salivary IL-38 concentration positively correlates with GCF IL-38 concentration.

# APPENDIX A

Table 1. The study visit procedure and the estimated time.

| Appointment / Procedures | Visit 1 | Estimated time |
|---|---|---|
| Informed Consent Form | X | 30-40 minutes |
| Demographics and medical history | X | |
| Evaluate eligibility and withdrawal criteria | X | |
| Periodontal clinical examination and radiographical evaluation | X | |
| Acquisition of Gingival crevicular fluid and saliva sample | X | 2-15 minutes |
| Payment | X | 1 minute |

Table 2. Scoring of indices.

| | 0 | 1 |
|---|---|---|
| Plaque Index | Plaque not present | Plaque present |
| Gingival Index | Gingival redness not present | Gingival redness present |
| Bleeding on Probing | No bleeding within 15 seconds of probing | Bleeding within 15 seconds of probing |
| Suppuration on Probing | No suppuration within 15 seconds of probing | Suppuration within 15 seconds of probing |
| Mobility | Not present | Present |

Table 3. Demographic and clinical parameters for periodontally healthy and periodontitis subjects.

| **Mean (± SD)<br>Median ($25^{th}$ – $75^{th}$ percentiles)** | **Healthy (n = 20)** | **Periodontitis (n = 20)** | **P-value** |
|---|---|---|---|
| **Age** | 51.40±12.13<br>12.13(42.0-63.80) | 50.40±12.73<br>50.50(42.25-59.50) | 0.801[a] |
| **Gender** | | | |
| **% Male (N)** | 35% (7) | 55% (11) | 0.204[b] |
| **% Female (N)** | 65% (13) | 45% (9) | |
| **Number of missing teeth** | 1.45±1.80<br>1.00(0.00-2.80) | 1.45±2.00<br>0.00(0.00-3.50) | 0.749[c] |
| **Probing depth (mm)** | 2.39±0.26<br>2.42(2.22-2.54) | 3.69±0.84<br>3.50(3.18-4.09) | **<0.001**[*c] |
| **Clinical Attachement level (mm)** | 0.69±0.85<br>0.20(0.036-1.21) | 3.76±1.66<br>3.53(2.85-4.37) | **<0.001**[*c] |
| **PI ( % )** | 16.18±19.07<br>11.80(0.00-25.90) | 59.23±35.95<br>56.10(28.30-100) | **<0.001**[*c] |

| | | | |
|---|---|---|---|
| **BOP ( % )** | 2.01±2.40<br>1.23(0.60-2.44) | 48.55±36.74<br>44.20(17.04-83.66) | **<0.001**[*c] |
| **Suppuration ( % )** | 0.00<br>0.00 | 4.44±9.23<br>0.30(0.00-6.10) | **<0.001**[*c] |
| **MOB ( % )** | 2.18±7.25<br>0.00 | 20.67±21.50<br>14.60(0.90-32.14) | **<0.001**[*c] |
| | | | |
| **% Ethnicity (N)** | | **Total** | **P-value** |
| **Not Hispanic or Latino** | 90% (18) | 80% (16) | 85% ( 34) | 0.695[d] |
| **Hispanic or Latino** | 5% (1) | 10% (2) | 7.5% (3) | |
| **Unknown/Not reported** | 5% (1) | 10% (2) | 7.5% (3) | |
| **% Race (N)** | | | | |
| **White** | 65% (13) | 50% (10) | 57.5% (23) | 0.088[d] |
| **Black/African American** | 0% | 25% (5) | 12.5% (5) | |
| **Native Hawaiian/Pacific Islander** | 0% | 0% | 0% | |
| **American Indian/Alaskan** | 0% | 0% | 0% | |
| **Asian** | 20%(4) | 15%(3) | 17.5%(7) | |
| **Multiple** | 0% | 5.0% (1) | 2.5%(1) | |
| **Don't Know** | 0% | 0% | 0% | |
| **No Answer/Other** | 15%(3) | 5.0% (1) | 10% (4) | |

* Statistically significant difference (P < .05).

a Determined by Independent-samples t-test.

b Determined by Chi-square test.

c Determined by Mann-Whitney U test.

d Determined by Fisher's exact test.

Table 4. Clinical parameters for all groups of sites of GCF.

| | | **Healthy (n = 20)** | **Periodontitis (n = 20)** | **Total** |
|---|---|---|---|---|
| **Arch:** | | | | |
| **Maxillary** | | 20 | 22 | 42 |
| **Mandibular** | | 20 | 18 | 38 |
| | | | | **P- Value** |
| **PD (mm)** | Mean (SD) | 2.85±0.36 | 7.57±1.44 | **<0.001**[*a] |
| | Median (25th - 75th percentiles) | 3.00 (3.00-3.00) | 7.00 (6.00-9.00) | |
| **CAL (mm)** | Mean (SD) | 0.57±1.15 | 7.90±2.19 | **<0.001**[*a] |
| | Median (25th - 75th percentiles) | 0.00 (0.00-0.75) | 8.00 (6.00-9.75) | |
| **PI ( % )** | | 25% | 70% | **0.005**[*a] |
| **BOP ( % )** | | 0% | 100% | **<0.001**[*a] |
| **Suppuration ( % )** | | 0% | 10% | **<0.001**[*a] |
| **MOB ( % )** | | 0% | 30% | **<0.001**[*a] |

* Statistically significant difference (P < .05).

a Determined by Generalized estimating equations (GEE)

Table 5. GCF volume mean levels ±SD for all groups of GCF collection.

| | Healthy (n = 20) | Periodontitis (n = 20) | P-value |
|---|---|---|---|
| **GCF volume** | | | |
| Mean ± SD | 1.20±0.58 | 2.11±0.50 | **<0.001*c** |
| Median (25th - 75th percentiles) | 1.00 (0.73-1.70) | 2.31 (1.80-2.50) | |

* Statistically significant difference (P < .05).

c Determined by Mann-Whitney U test.

Table 6: Frequency of detection of IL-1β, IL-17, IL-36β, IL-37, IL-38 in GCF.

| % sites (N) positive for | Healthy (n = 20) | Periodontitis (n = 20) | P-Value |
|---|---|---|---|
| **IL-1β** | 100% (20) | 100% (20) | 1.000[d] |
| **IL-17** | 50% (10) | 100% (20) | **<0.001*d** |
| **IL-36β** | 100% (20) | 100% (20) | 1.000[d] |
| **IL-37** | 95% (19) | 95% (19) | 1.000[d] |
| **IL-38** | 85% (17) | 60% (12) | 0.150[d] |

* Statistically significant difference (P < .05).

d Determined by Fisher's exact test.

Table 7: Frequency of detection of IL-1β, IL-17, IL-36β, IL-37, IL-38 in Saliva.

| % sites (N) positive for | Healthy (n = 20) | Periodontitis (n = 20) | P-Value |
|---|---|---|---|
| **IL-1β** | 100% (20) | 100% (20) | 1.000[d] |
| **IL-17** | 20% (4) | 20% (4) | 1.000[d] |
| **IL-36β** | 100% (20) | 100% (20) | 1.000[d] |
| **IL-37** | 95% (19) | 95% (19) | 1.000[d] |
| **IL-38** | 75% (15) | 50% (10) | 0.190[d] |

* Statistically significant difference (P < .05).

d Determined by Fisher's exact test.

Table 8: GCF amount (pg) of IL-1β, IL-17, IL-36β, IL-37, IL-38.

| Interleukin | | Healthy | Periodontitis | P-value |
|---|---|---|---|---|
| **IL-1β** | Mean (SD) | 44.20±59.06 | 383.22±388.8 | **<0.001*a** |

| | | | | |
|---|---|---|---|---|
| | Median (25th - 75th percentiles) | 20.02 (5.88-49.41) | 284.8 (159.59-449.09) | **<0.001*b** |
| **IL-17** | Mean (SD) | 0.42±0.50 | 2.75±1.84 | **<0.001*a** |
| | Median (25th - 75th percentiles) | 0.19 (0.058-0.67) | 2.50 (1.11-3.92) | **<0.001*b** |
| **IL-36β** | Mean (SD) | 184.45±89.28 | 224.18±99.08 | 0.191[a] |
| | Median (25th - 75th percentiles) | 174.82 (121.1-212.2) | 219.8 (132.79-304.31) | 0.157[b] |
| **IL-37** | Mean (SD) | 62.63±70.06 | 57.93±52.16 | 0.889[a] |
| | Median (25th - 75th percentiles) | 49.8 (22.91-72.82) | 40.05 (20.48-88.73) | 0.455[b] |
| **IL-38** | Mean (SD) | 10.77 ± 6.94 | 11.36 ±12.17 | 0.611[a] |
| | Median (25th - 75th percentiles) | 10.58 (6.33-15.2) | 7.76 (0.78-20.2) | 0.269[b] |

* Statistically significant difference (P < .05).

a Determined by Mann-Whitney U test

b Adjusted for age and gender

Table 9: GCF concentrations (pg/µl) of IL-1β, IL-17, IL-36β, IL-37, IL-38.

| Interleukin | | Healthy | Periodontitis | P-value |
|---|---|---|---|---|
| **IL-1β** | Mean (SD) | 37.69±49.49 | 183.70±173.29 | **<0.001*a** |
| | Median (25th - 75th percentiles) | 18.51 (4.64-54.63) | 132.01 (73.30-244.47) | **<0.001*a** |
| **IL-17** | Mean (SD) | 0.455±0.62 | 1.34 ±0.89 | **<0.001*a** |
| | Median (25th - 75th percentiles) | 0.23 (0.47-0.67) | 1.24 (1.24-2.05) | **0.002*b** |
| **IL-36β** | Mean (SD) | 182.86 ± 117.79 | 124.94±96.90 | **0.049*a** |
| | Median (25th - 75th percentiles) | 158(93.80-244.18) | 89.8(58.87-159.86) | 0.088[b] |
| **IL-37** | Mean (SD) | 60.69±75.63 | 30.16±31.06 | **0.046*a** |
| | Median (25th - 75th percentiles) | 37.44 (24.55-55.01 | 18.14 (10.58-46.18) | **0.017*b** |
| **IL-38** | Mean (SD) | 10.54 ±7.90 | 4.99±4.91 | **0.012*a** |
| | Median (25th - 75th percentiles) | 8.66 (4.93-13.99) | 3.93 ( 0.43-8.60) | **0.011*b** |

* Statistically significant difference (P < .05).

a Determined by Mann-Whitney U test

b Adjusted for age and gender

Table 10: GCF levels of Pro-inflammatory interleukins (IL-1β, IL-17, IL-36β) and Anti-inflammatory interleukins (IL-37, IL-38) in (pg/µl).

| Index | | Healthy (n=20) | Periodontitis (n=20) | P-value |
|---|---|---|---|---|
| **Pro-inflammatory (pg/µl)** | Mean ± SD | 5.53±4.80 | 20.23±23.55 | **0.006*a** |
| | Median (25th - 75th percentiles) | 3.90 (2.14-8.20) | 11.16 (4.22-23.43) | |
| **Anti-inflammatory (pg/µl)** | Mean ± SD | 0.46±0.59 | 0.16±0.18 | **0.006*a** |
| | Median (25th - 75th percentiles) | 0.25 (0.12-0.46) | 0.09 (0.04-0.23) | |

* Statistically significant difference (P < .05).

a Determined by Mann-Whitney U test

Table 11: Association between frequency of detection of interleukins in GCF with GCF volume (µl) and PD of site of sample collection.

| Interleukin[e] | | Detected | Not detected | P |
|---|---|---|---|---|
| IL-17 | N | 30 | 10 | 0.129[a] |
| | **Volume (µl)** | | | |
| | Mean (SD) | 1.74±0.67 | 1.37±0.64 | |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 1.80 (1.12-2.60) | 1.16 (0.80-1.10) | |
| | **Mean PD** | | | |
| | Mean (SD) | 6.00±6.50 | 5.85±0.24 | **<0.001*[a]** |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 6.50 (3.00-8.13) | 3.00 (2.50-3.00) | |
| IL-37 | N | 38 | 2 | 0.908[a] |
| | **Volume (µl)** | | | |
| | Mean (SD) | 1.70±0.68 | 1.64±0.84 | |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 1.64 (0.10-42.50) | 0.90 (1.04-2.70) | |
| | **Mean PD** | | | 0.864[a] |
| | Mean (SD) | 5.20±2.52 | 5.50±4.94 | |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 4.30 (3.00-7.12) | 5.00 (NA) | |
| IL-38 | N | 29 | 11 | 0.440[a] |
| | **Volume (µl)** | | | |
| | Mean (SD) | 1.60±0.72 | 1.80±0.58 | |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 1.67 (0.90-2.40) | 1.81 (1.20-2.23) | |
| | **Mean PD** | | | 0.104[a] |
| | Mean (SD) | 4.80±2.52 | 6.40±2.50 | |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 3.00 (3.00-7.00) | 7.00 (3.00-9.00) | |

* Statistically significant difference (P < .05).

a Determined by Mann-Whitney U test

e This test could not be conducted for IL-1β and IL-36β as they exhibited 100% detection.

NA The interquartile range could not be determined because the sample size is low.

Table 12: Spearman's correlation coefficient between GCF interleukins amount (pg) with GCF volume (µl) and mean PD of sites of sample collection.

| Interleukin | | Volume (µl) | Mean PD |
|---|---|---|---|
| IL-1β | rho | 0.559 | 0.747 |
| | p-value | **<0.001*** | **<0.001*** |
| IL-17 | rho | 0.519 | 0.725 |
| | p-value | **<0.001*** | **<0.001*** |

| | | | |
|---|---|---|---|
| **IL-36** | rho | 0.065 | 0.189 |
| | p-value | 0.680 | 0.240 |
| **IL-37** | rho | 0.010 | -0.025 |
| | p-value | 0.950 | 0.917 |
| **IL-38** | rho | 0.182 | -0.066 |
| | p-value | 0.260 | 0.685 |

* Statistically significant difference (P < .05).

Table 13: Spearman's correlation coefficient between GCF interleukins concentration (pg/µl) with GCF volume (µl) and with mean PD of sites of sample collection.

| **Interleukin** | | **Volume (µl)** | **Mean PD** |
|---|---|---|---|
| **IL-1β** | rho | 0.388 | 0.658 |
| | p-value | **0.013*** | **<0.001*** |
| **IL-17** | rho | 0.196 | 0.576 |
| | p-value | 0.226 | **<0.001*** |
| **IL-36** | rho | -0.701 | -0.344 |
| | p-value | **<0.001*** | **0.03*** |
| **IL-37** | rho | -0.437 | -0.430 |
| | p-value | **0.005*** | **0.006*** |
| **IL-38** | rho | -0.322 | -0.376 |
| | p-value | **0.042*** | **0.017*** |

* Statistically significant difference (P < .05).

Table 14: Spearman's correlation coefficient between GCF interleukins.

| **Interluekin** | | **IL-1B** | **IL-17** | **IL-36B** | **IL-37** | **IL-38** |
|---|---|---|---|---|---|---|
| **IL-1β** | rho | 1 | 0.77 | -0.096 | -0.265 | -0.264 |
| | p-value | - | **<0.001** | 0.555 | 0.098 | 0.099 |
| **IL-17** | rho | 0.77 | 1 | 0.097 | -0.335 | -0.26 |
| | p-value | **<0.001** | - | 0.552 | **0.035** | 0.105 |
| **IL-36β** | rho | -0.096 | 0.097 | 1 | 0.204 | 0.162 |
| | p-value | 0.555 | 0.552 | - | 0.207 | 0.317 |
| **IL-37** | rho | -0.265 | -0.335 | 0.204 | 1 | 0.261 |
| | p-value | 0.098 | **0.035** | 0.207 | - | 0.103 |
| **IL-38** | rho | -0.264 | -0.26 | 0.162 | 0.261 | 1 |
| | p-value | 0.099 | 0.105 | 0.317 | 0.103 | - |

* Statistically significant difference (P < .05).

Table 15: Salivary concentrations (pg/ml) of IL-1β, IL-17, IL-36β, IL-37, IL-38.

| Interleukin | | Healthy | Periodontitis | P-value |
|---|---|---|---|---|
| **IL-1β** | Mean (SD) | 17.91±28.2 | 45.70±70.13 | 0.052[a] **0.0290**[*b] **0.005**[*c] |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 2.20 (0.60-26.04) | 6.90 (2.92-81.6) | |
| **IL-17** | Mean (SD) | 0.80±2.20 | 0.14±0.41 | |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 0 | 0 | |
| **IL-36β** | Mean (SD) | 653.4±466.8 | 691.40±455.04 | 0.620[a] 0.530[b] 0.410[c] |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 612.6 (277.50-989.40) | 594.8 (352.83-838.32) | |
| **IL-37** | Mean (SD) | 44.61±55.8 | 28.41±40.06 | 0.607[a] 0.418[b] 0.412[c] |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 21.94 (3.26-75.1) | 10.2 (4.32-43.91) | |
| **IL-38** | Mean (SD) | 41.20±48.30 | 24.61±39.30 | 0.106[a] 0.139[b] 0.092[c] |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 24.3 (0.77-70.91) | 5.90 (0.38-38.91) | |

* Statistically significant difference (P < .05).

a Determined by Mann-Whitney U test.

b Adjusted for age and gender.

c Adjusted for age and gender and saliva weight.

Table 16: Salivary Concentrations of Pro-inflammatory interleukins (IL-1β, IL-17, IL-36β) and Anti-inflammatory interleukins (IL-37, IL-38).

| Index | | Healthy (n=20) | Periodontitis (n=20) | P-value |
|---|---|---|---|---|
| **Pro-inflammatory** | Mean ± SD | 38.2±98.12 | 100.5±303.11 | 0.068[a] |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 9.10 (4.60-16.6) | 26.53 (5.93-48.61) | |
| **Anti-inflammatory** | Mean ± SD | 0.27±0.55 | 0.103±0.144 | 0.068[a] |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 0.10 (0.06-0.22) | 0.037 (0.02-0.16) | |

* Statistically significant difference (P < .05).

a Determined by Mann-Whitney U test.

Table 17: Association between calculated saliva weight and detection frequency.

| Interleukin[e] | Calculated saliva weight | Detected | Not detected | P |
|---|---|---|---|---|
| **IL-17** | N | 8 | 32 | **0.018**[*a] |
| | Mean (SD) | 3.04±1.20 | 5.90±3.23 | |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 3.01 (2.03-4.30) | 4.30 (3.60-8.12) | |

| | | | | |
|---|---|---|---|---|
| **IL-37** | N | 38 | 2 | 0.974[a] |
| | Mean (SD) | 5.01±3.05 | 6.30±5.29 | |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 4.06 (3.50-5.01) | 6.30 (NA) | |
| **IL-38** | N | 25 | 15 | 0.639[a] |
| | Mean (SD) | 4.98±3.22 | 5.23±3.01 | |
| | Median ($25^{th}$ - $75^{th}$ percentiles) | 10.37 (3.27-6.40) | 4.50 (3.40-5.21) | |

* Statistically significant difference (P < .05).

a Determined by Mann-Whitney U test

e This test could not be conducted for IL-1β and IL-36β as they exhibited 100% detection.

NA The interquartile range could not be determined because the sample size is low.

Table 18: Spearman's correlation coefficient between salivary levels of interleukins (pg/ml) and salivary weight.

| | **Interleukins** | **IL-1β** | **IL-17** | **IL-36β** | **IL-37** | **IL-38** |
|---|---|---|---|---|---|---|
| **Calculated saliva weight** | rho | -0.251 | -0.348 | -0.250 | -0.095 | 0.138 |
| | p-value | 0.118 | **0.028*** | 0.120 | 0.562 | 0.394 |

* Statistically significant difference (P < .05).

Table 19: Spearman's correlation coefficient between calculated interleukins concentration levels in Saliva (pg/ml) and GCF (pg/µl).

| **Saliva mediators** | **GCF mediators** | **Correlation Coefficient** | **P-value** |
|---|---|---|---|
| **IL-1β** | IL-1B | 0.574 | **<0.001*** |
| **IL-17** | IL-17 | 0.067 | 0.681 |
| **IL-36β** | IL36b | 0.051 | 0.754 |
| **IL-37** | IL-37 | -0.037 | 0.821 |
| **IL-38** | IL-38 | 0.536 | **<0.001*** |

* Statistically significant difference (P < .05).

# APPENDIX B

Figure 1: Study Schema

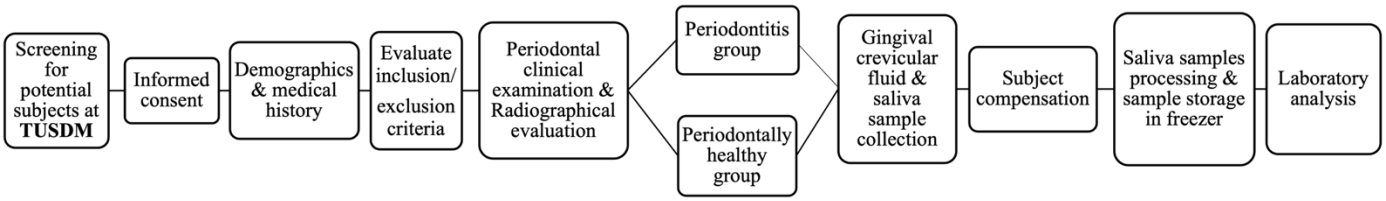


Figure 2. Box-plot of GCF volume of healthy and periodontitis subjects.

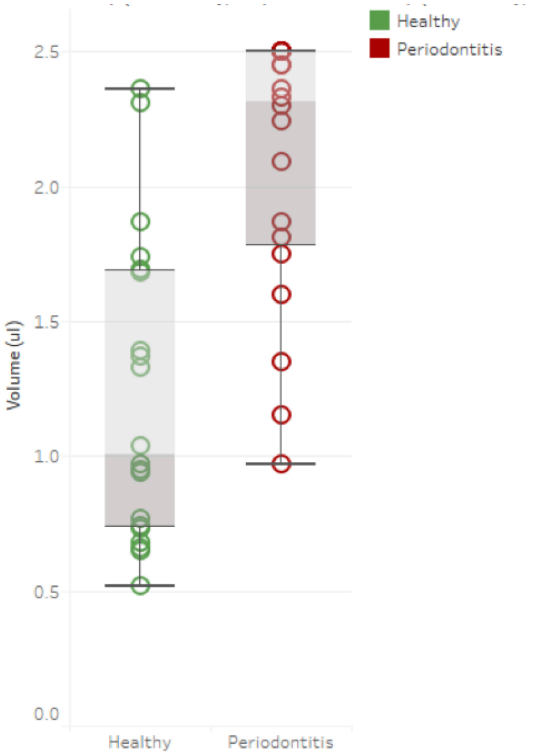


Figure 3: Bar chart of frequency of detection of IL-1β, IL-17, IL-36β, IL-37, IL-38 in GCF.

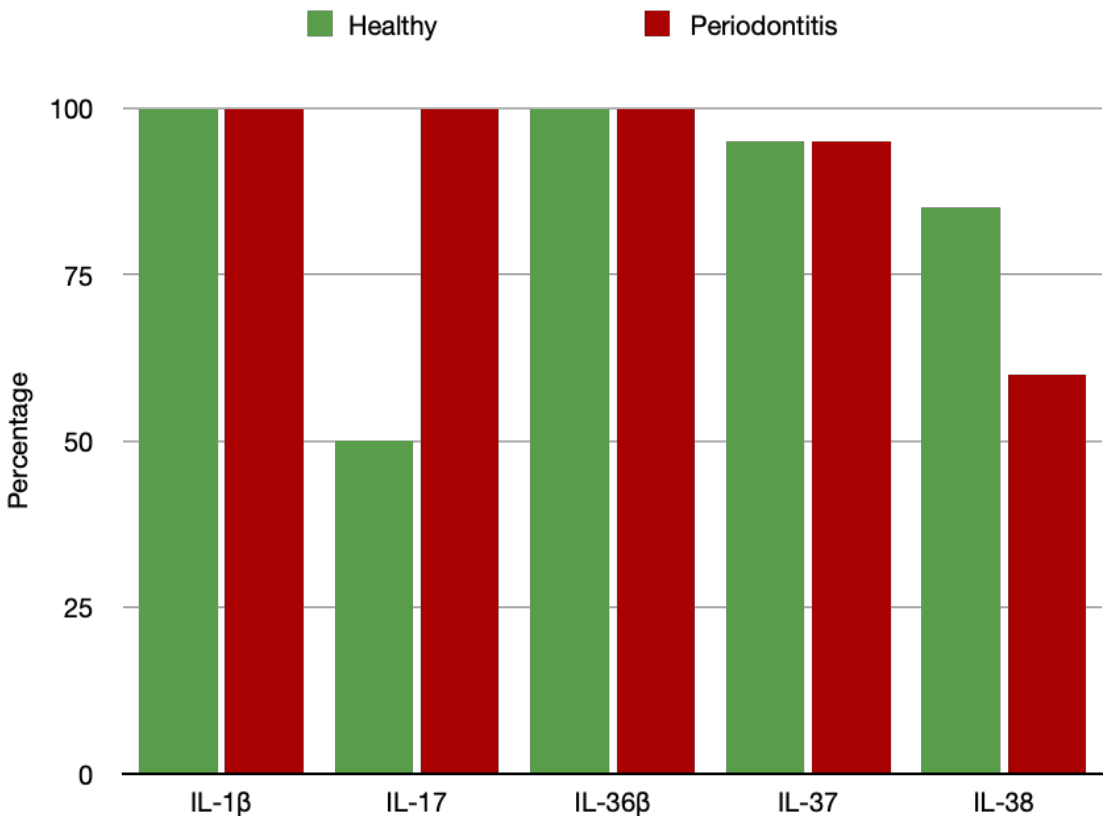


Figure 4: Bar chart of frequency of detection of IL-1β, IL-17, IL-36β, IL-37, IL-38 in saliva.

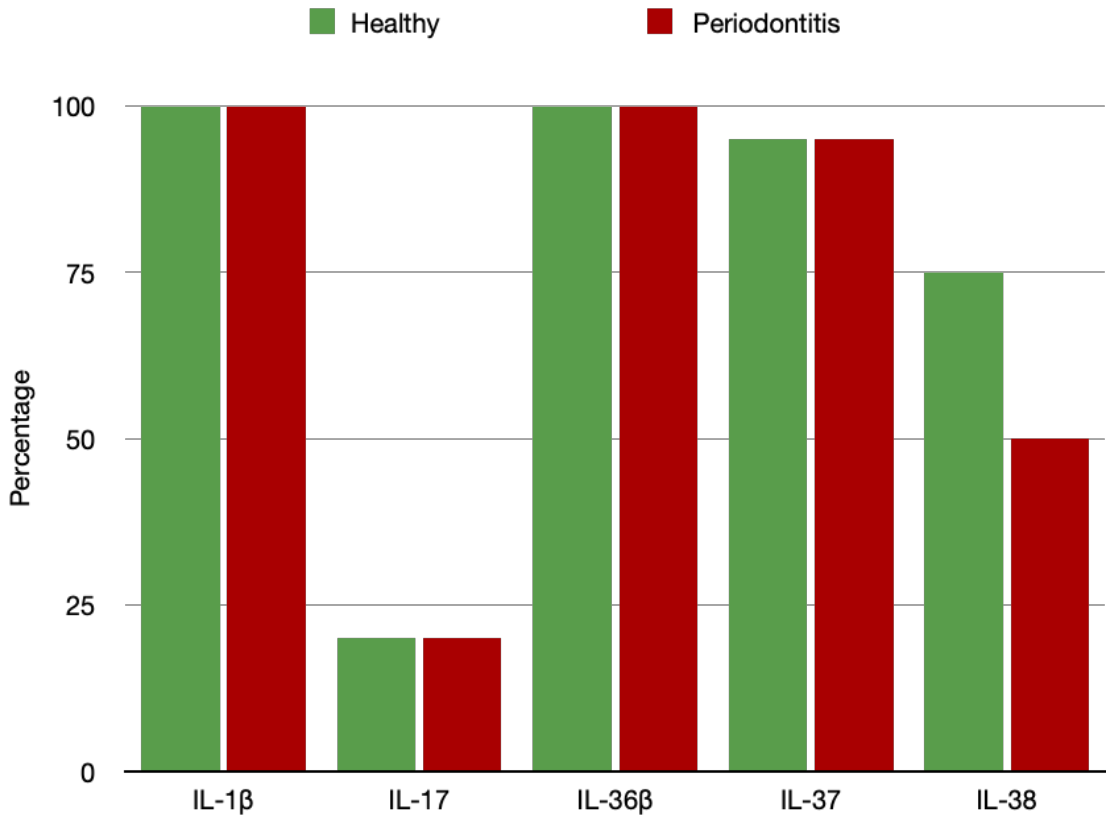


Figure 5: Bar chart of amount (pg) of IL-1β, IL-17, IL-36β, IL-37, IL-38 in GCF of healthy and periodontitis subjects.

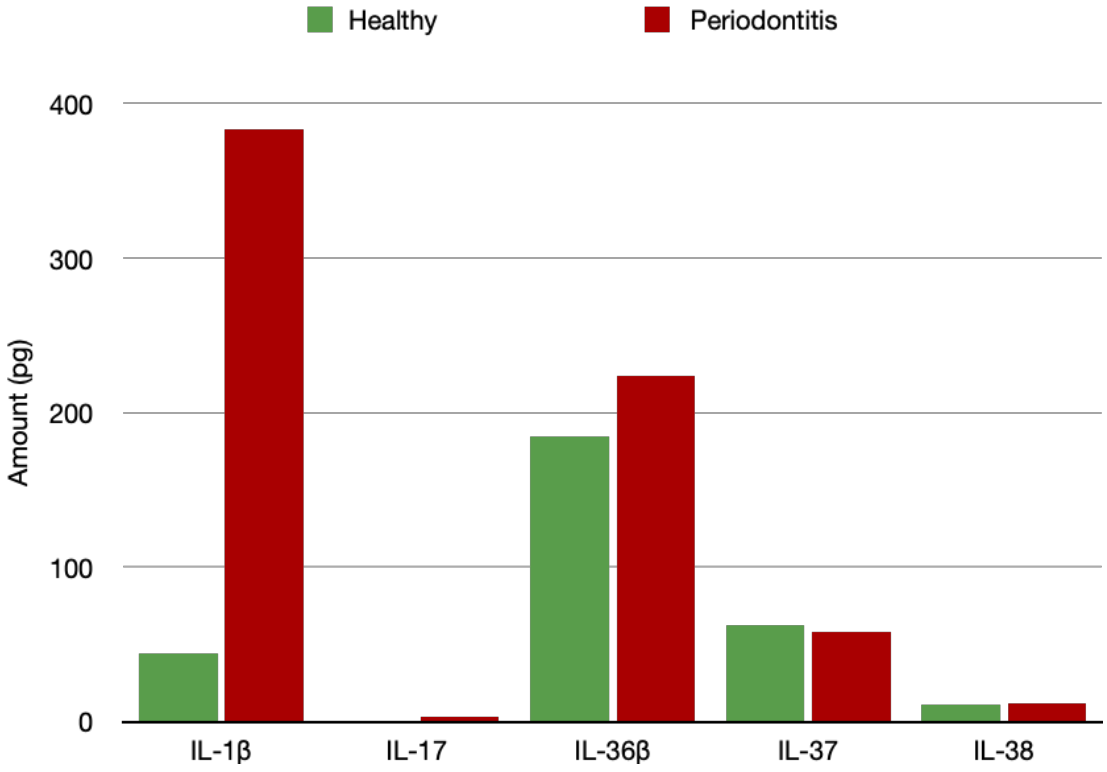


Figure 6: Bar chart of concentrations (pg/ µl) of IL-1β, IL-17, IL-36β, IL-37, IL-38 in GCF of healthy and periodontitis subjects.

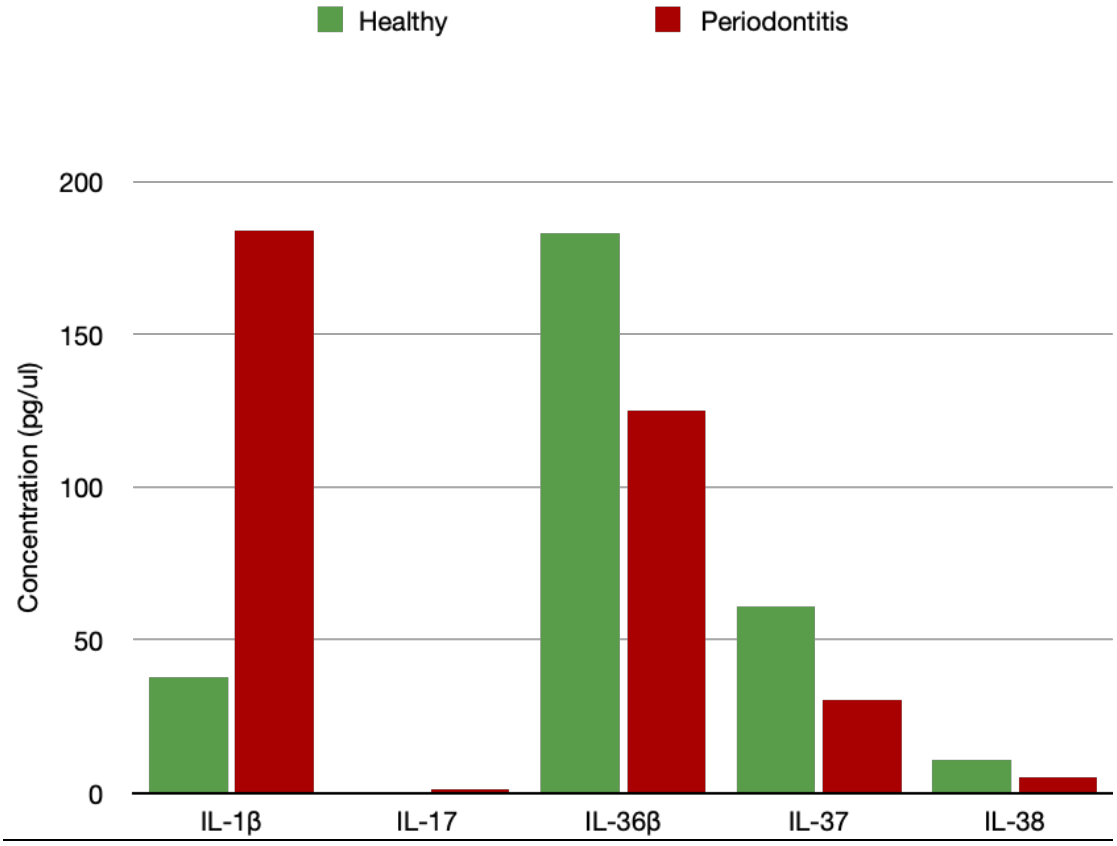


Figure 7: Box-plot of GCF concentrations (pg/ µl) of IL-1β, IL-17, IL-36β, IL-37, IL-38 in GCF of healthy and periodontitis subjects.

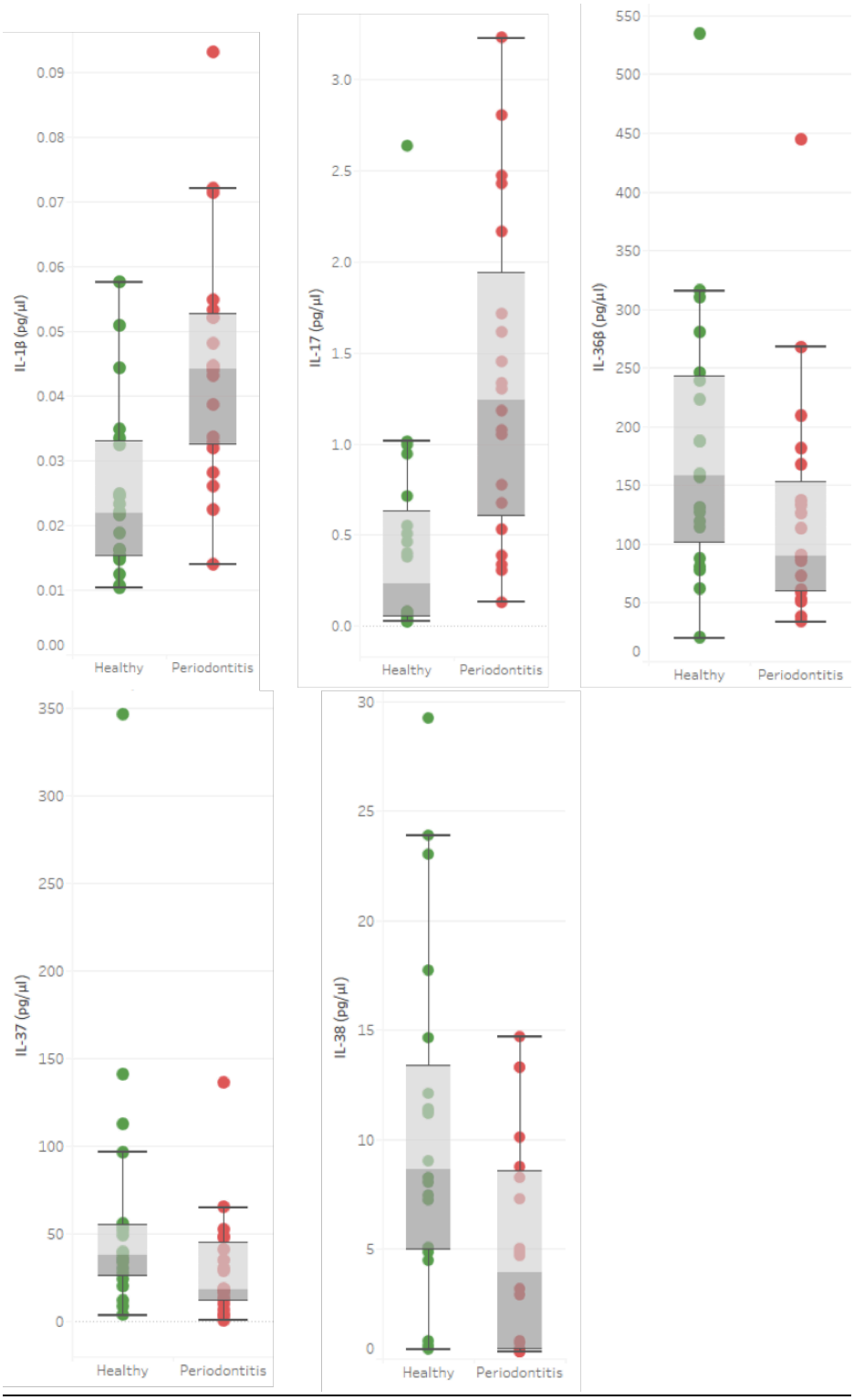


Figure 8: Pie-chart of pro-inflammatory index and anti-inflammatory index ratios in GCF of healthy and periodontitis subjects.

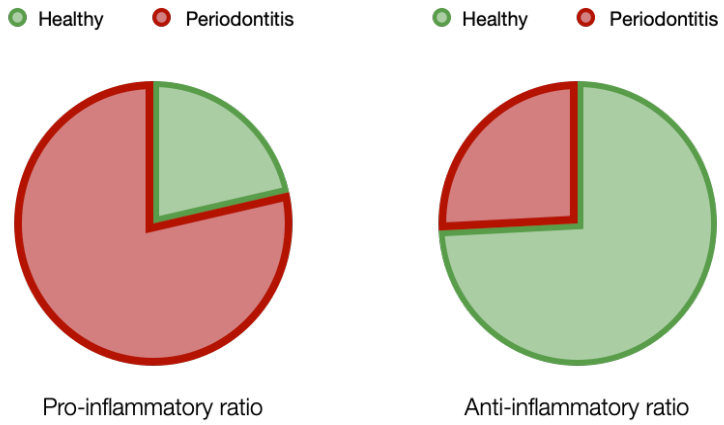



Figure 9: Box-plot of pro-inflammatory index and anti-inflammatory index ratios in GCF of healthy and periodontitis subjects.

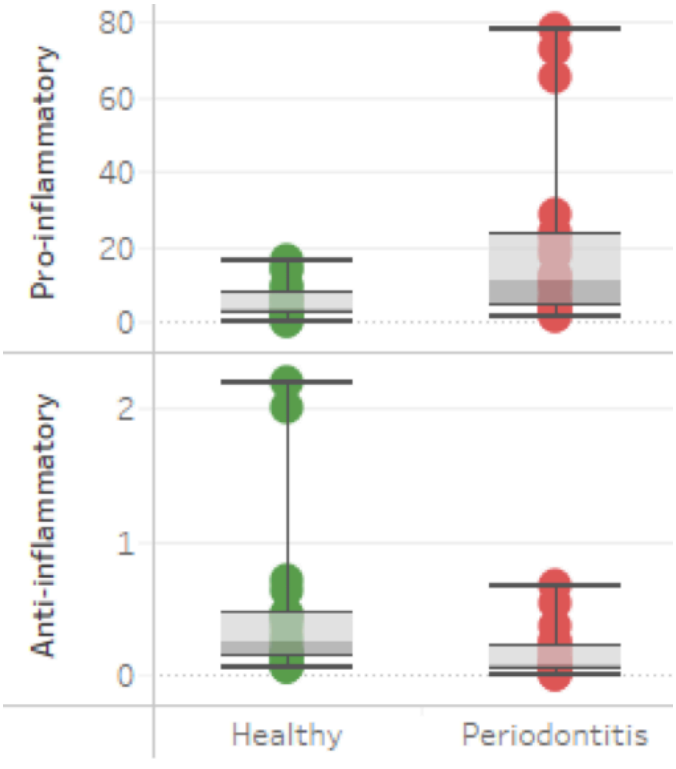


Figure 10: Bar chart of concentrations (pg/ml) of IL-1β, IL-17, IL-36β, IL-37, and IL-38 in the saliva of periodontitis patients and healthy subjects.

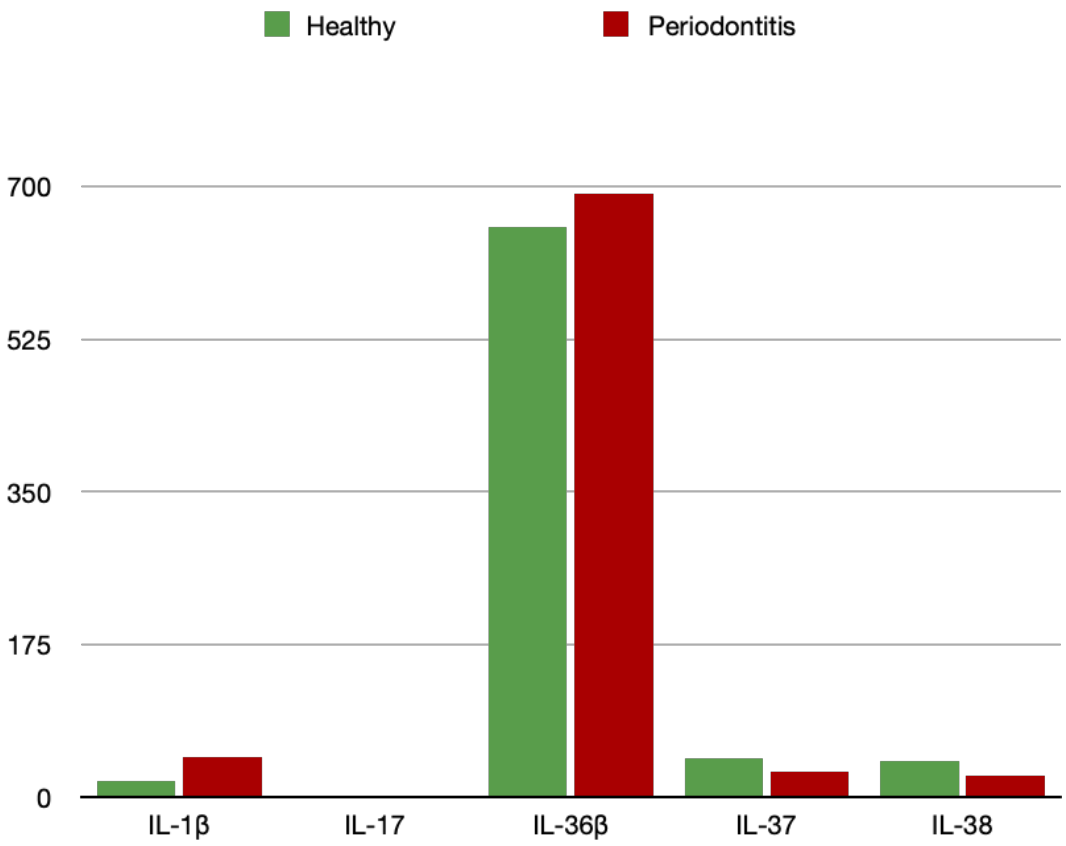


Figure 11: Pie-charts of Pro-inflammatory index and Anti-inflammatory index ratios in the saliva of healthy and periodontitis subjects.

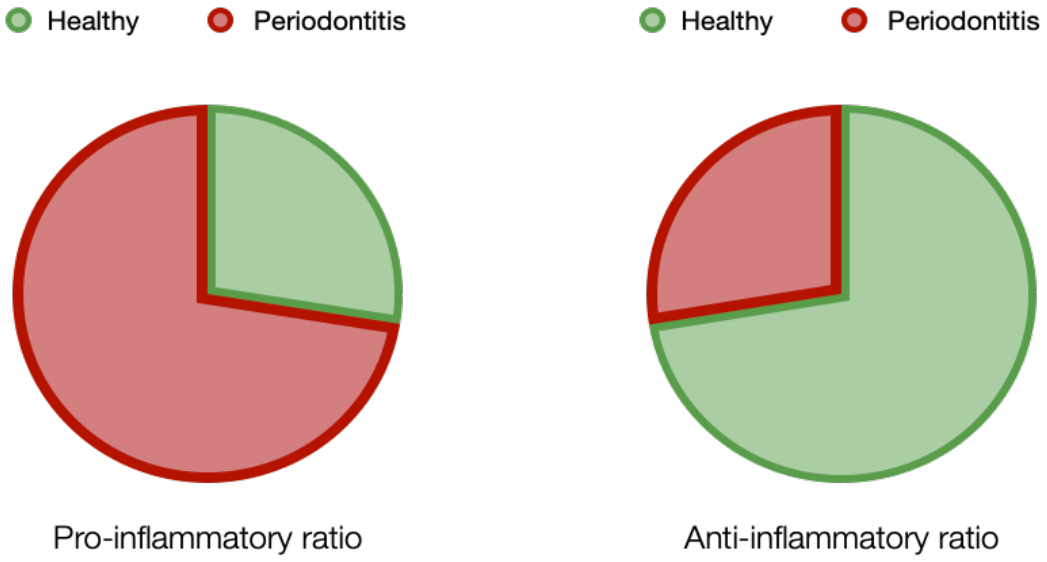
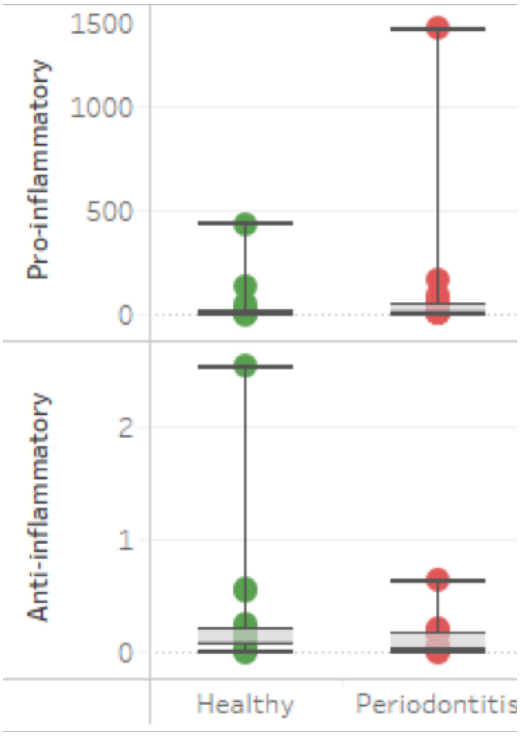



Figure 12: Box-plots of Pro-inflammatory index and Anti-inflammatory Index ratios in the saliva of healthy and periodontitis subjects.



## BIBLIOGRAPHY


1. Papapanou PN, Sanz M, Buduneli N, et al. Periodontitis: Consensus report of workgroup 2 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. *J Periodontol*. Jun 2018;89 Suppl 1:S173-S182. doi:10.1002/JPER.17-0721
2. Socransky SS, Haffajee AD. The Bacterial Etiology of Destructive Periodontal Disease: Current Concepts. *J Periodontol*. Apr 1992;63 Suppl 4S:322-331. doi:10.1902/jop.1992.63.4s.322
3. Eke PI, Dye BA, Wei L, et al. Update on Prevalence of Periodontitis in Adults in the United States: NHANES 2009 to 2012. *J Periodontol*. May 2015;86(5):611-22. doi:10.1902/jop.2015.140520
4. Eke PI, Thornton-Evans GO, Wei L, Borgnakke WS, Dye BA, Genco RJ. Periodontitis in US Adults: National Health and Nutrition Examination Survey 2009-2014. *J Am Dent Assoc*. Jul 2018;149(7):576-588 e6. doi:10.1016/j.adaj.2018.04.023
5. Cochran DL. Inflammation and bone loss in periodontal disease. *J Periodontol*. Aug 2008;79(8 Suppl):1569-76. doi:10.1902/jop.2008.080233
6. Papathanasiou E, Conti P, Carinci F, Lauritano D, Theoharides TC. IL-1 Superfamily Members and Periodontal Diseases. *J Dent Res*. Dec 2020;99(13):1425-1434. doi:10.1177/0022034520945209
7. Salvi GE, Lang NP. Host response modulation in the management of periodontal diseases. *J Clin Periodontol*. 2005;32 Suppl 6:108-29. doi:10.1111/j.1600-051X.2005.00785.x
8. Loos BG, Van Dyke TE. The role of inflammation and genetics in periodontal disease. *Periodontol 2000*. Jun 2020;83(1):26-39. doi:10.1111/prd.12297
9. Cekici A, Kantarci A, Hasturk H, Van Dyke TE. Inflammatory and immune pathways in the pathogenesis of periodontal disease. *Periodontol 2000*. Feb 2014;64(1):57-80. doi:10.1111/prd.12002
10. Darveau RP. Periodontitis: a polymicrobial disruption of host homeostasis. *Nat Rev Microbiol*. Jul 2010;8(7):481-90. doi:10.1038/nrmicro2337
11. Garlet GP. Destructive and protective roles of cytokines in periodontitis: a re-appraisal from host defense and tissue destruction viewpoints. *J Dent Res*. Dec 2010;89(12):1349-63. doi:10.1177/0022034510376402
12. Kinane DF, Preshaw PM, Loos BG, Working Group 2 of Seventh European Workshop on P. Host-response: understanding the cellular and molecular mechanisms of host-microbial interactions--consensus of the Seventh European Workshop on Periodontology. *J Clin Periodontol*. Mar 2011;38 Suppl 11:44-8. doi:10.1111/j.1600-051X.2010.01682.x
13. Van Dyke TE, van Winkelhoff AJ. Infection and inflammatory mechanisms. *J Periodontol*. Apr 2013;84(4 Suppl):S1-7. doi:10.1902/jop.2013.1340018
14. Barros SP, Williams R, Offenbacher S, Morelli T. Gingival crevicular fluid as a source of biomarkers for periodontitis. *Periodontol 2000*. Feb 2016;70(1):53-64. doi:10.1111/prd.12107
15. Giannobile WV, Beikler T, Kinney JS, Ramseier CA, Morelli T, Wong DT. Saliva as a diagnostic tool for periodontal disease: current state and future directions. *Periodontol 2000*. 2009;50:52-64. doi:10.1111/j.1600-0757.2008.00288.x
16. Zhang Y, Sun J, Lin CC, Abemayor E, Wang MB, Wong DT. The emerging landscape of salivary diagnostics. *Periodontol 2000*. Feb 2016;70(1):38-52. doi:10.1111/prd.12099

17. Arias-Bujanda N, Regueira-Iglesias A, Balsa-Castro C, Nibali L, Donos N, Tomas I. Accuracy of single molecular biomarkers in saliva for the diagnosis of periodontitis: A systematic review and meta-analysis. *J Clin Periodontol*. Jan 2020;47(1):2-18. doi:10.1111/jcpe.13202
18. Kumar H, Kawai T, Akira S. Pathogen recognition by the innate immune system. *Int Rev Immunol*. Feb 2011;30(1):16-34. doi:10.3109/08830185.2010.529976
19. Graves DT, Cochran D. The contribution of interleukin-1 and tumor necrosis factor to periodontal tissue destruction. *J Periodontol*. Mar 2003;74(3):391-401. doi:10.1902/jop.2003.74.3.391
20. Stadler AF, Angst PD, Arce RM, Gomes SC, Oppermann RV, Susin C. Gingival crevicular fluid levels of cytokines/chemokines in chronic periodontitis: a meta-analysis. *J Clin Periodontol*. Sep 2016;43(9):727-45. doi:10.1111/jcpe.12557
21. Gu C, Wu L, Li X. IL-17 family: cytokines, receptors and signaling. *Cytokine*. Nov 2013;64(2):477-85. doi:10.1016/j.cyto.2013.07.022
22. Dinarello CA. Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. *Blood*. Apr 7 2011;117(14):3720-32. doi:10.1182/blood-2010-07-273417
23. Vernal R, Dutzan N, Chaparro A, Puente J, Antonieta Valenzuela M, Gamonal J. Levels of interleukin-17 in gingival crevicular fluid and in supernatants of cellular cultures of gingival tissue from patients with chronic periodontitis. *J Clin Periodontol*. Apr 2005;32(4):383-9. doi:10.1111/j.1600-051X.2005.00684.x
24. Vahabi S, Yadegari Z, Pournaghi S. The comparison of the salivary concentration of interleukin-17 and interleukin-18 in patients with chronic periodontitis and healthy individuals. *Dent Res J (Isfahan)*. Jul-Aug 2020;17(4):280-286.
25. Yang X, Li C, Pan Y. The Influences of Periodontal Status and Periodontal Pathogen Quantity on Salivary 8-Hydroxydeoxyguanosine and Interleukin-17 Levels. *J Periodontol*. May 2016;87(5):591-600. doi:10.1902/jop.2015.150390
26. Chen WJ, Yu X, Yuan XR, et al. The Role of IL-36 in the Pathophysiological Processes of Autoimmune Diseases. *Front Pharmacol*. 2021;12:727956. doi:10.3389/fphar.2021.727956
27. Kursunlu SF, Ozturk VO, Han B, Atmaca H, Emingil G. Gingival crevicular fluid interleukin-36beta (-1F8), interleukin-36gamma (-1F9) and interleukin-33 (-1F11) levels in different periodontal disease. *Arch Oral Biol*. Jan 2015;60(1):77-83. doi:10.1016/j.archoralbio.2014.08.021
28. Dinarello CA. Overview of the IL-1 family in innate inflammation and acquired immunity. *Immunol Rev*. Jan 2018;281(1):8-27. doi:10.1111/imr.12621
29. Offenbacher S, Jiao Y, Kim SJ, et al. GWAS for Interleukin-1beta levels in gingival crevicular fluid identifies IL37 variants in periodontal inflammation. *Nat Commun*. Sep 11 2018;9(1):3686. doi:10.1038/s41467-018-05940-9
30. Saglam M, Koseoglu S, Savran L, Pekbagriyanik T, Saglam G, Sutcu R. Levels of interleukin-37 in gingival crevicular fluid, saliva, or plasma in periodontal disease. *J Periodontal Res*. Oct 2015;50(5):614-21. doi:10.1111/jre.12241
31. Han MM, Yuan XR, Shi X, et al. The Pathological Mechanism and Potential Application of IL-38 in Autoimmune Diseases. *Front Pharmacol*. 2021;12:732790. doi:10.3389/fphar.2021.732790
32. van de Veerdonk FL, de Graaf DM, Joosten LA, Dinarello CA. Biology of IL-38 and its role in disease. *Immunol Rev*. Jan 2018;281(1):191-196. doi:10.1111/imr.12612

33. Luo D, Chen Y, Zhou N, Li T, Wang H. Blockade of Th17 response by IL-38 in primary Sjogren's syndrome. *Mol Immunol*. Nov 2020;127:107-111. doi:10.1016/j.molimm.2020.09.006
34. Korkmaz H, Hatipoglu M, Kayar NA. Interleukin-38: A crucial player in periodontitis. *Oral Dis*. Jul 16 2023;doi:10.1111/odi.14657
35. Zarrabi M, Nazarinia M, Rahimi Jaberi A, Gholijani N, Amirghofran Z. Elevated IL-38 Serum Levels in Newly Diagnosed Multiple Sclerosis and Systemic Sclerosis Patients. *Med Princ Pract*. 2021;30(2):146-153. doi:10.1159/000510915
36. Goodson JM. Diagnosis of Periodontitis by Physical Measurement: Interpretation From Episodic Disease Hypothesis. *J Periodontol*. Apr 1992;63 Suppl 4S:373-382. doi:10.1902/jop.1992.63.4s.373
37. Kornman KS. Contemporary approaches for identifying individual risk for periodontitis. *Periodontol 2000*. Oct 2018;78(1):12-29. doi:10.1111/prd.12234
38. Paque PN, Hjerppe J, Zuercher AN, Jung RE, Joda T. Salivary biomarkers as key to monitor personalized oral healthcare and precision dentistry: A scoping review. *Front Oral Health*. 2022;3:1003679. doi:10.3389/froh.2022.1003679
39. Korte DL, Kinney J. Personalized medicine: an update of salivary biomarkers for periodontal diseases. *Periodontol 2000*. Feb 2016;70(1):26-37. doi:10.1111/prd.12103
40. Papapanou PN, Sanz M, Buduneli N, et al. Periodontitis: Consensus report of workgroup 2 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. *J Clin Periodontol*. Jun 2018;45 Suppl 20:S162-S170. doi:10.1111/jcpe.12946
41. Chapple IL, Landini G, Griffiths GS, Patel NC, Ward RS. Calibration of the Periotron 8000 and 6000 by polynomial regression. *J Periodontal Res*. Feb 1999;34(2):79-86. doi:10.1111/j.1600-0765.1999.tb02226.x
42. Papathanasiou E, Teles F, Griffin T, et al. Gingival crevicular fluid levels of interferon-gamma, but not interleukin-4 or -33 or thymic stromal lymphopoietin, are increased in inflamed sites in patients with periodontal disease. *J Periodontal Res*. Feb 2014;49(1):55-61. doi:10.1111/jre.12078
43. Khalifian S, Raimondi G, Brandacher G. The use of luminex assays to measure cytokines. *J Invest Dermatol*. Apr 2015;135(4):1-5. doi:10.1038/jid.2015.36
44. Do KT, Wahl S, Raffler J, et al. Characterization of missing values in untargeted MS-based metabolomics data and evaluation of missing data handling strategies. *Metabolomics*. Sep 20 2018;14(10):128. doi:10.1007/s11306-018-1420-2
45. Xia J, Wishart DS. Web-based inference of biological patterns, functions and pathways from metabolomic data using MetaboAnalyst. *Nat Protoc*. Jun 2011;6(6):743-60. doi:10.1038/nprot.2011.319
46. Lazar C, Gatto L, Ferro M, Bruley C, Burger T. Accounting for the Multiple Natures of Missing Values in Label-Free Quantitative Proteomics Data Sets to Compare Imputation Strategies. *J Proteome Res*. Apr 1 2016;15(4):1116-25. doi:10.1021/acs.jproteome.5b00981
47. Nazar Majeed Z, Philip K, Alabsi AM, Pushparajan S, Swaminathan D. Identification of Gingival Crevicular Fluid Sampling, Analytical Methods, and Oral Biomarkers for the Diagnosis and Monitoring of Periodontal Diseases: A Systematic Review. *Dis Markers*. 2016;2016:1804727. doi:10.1155/2016/1804727
48. Guentsch A, Kramesberger M, Sroka A, Pfister W, Potempa J, Eick S. Comparison of gingival crevicular fluid sampling methods in patients with severe chronic periodontitis. *J Periodontol*. Jul 2011;82(7):1051-60. doi:10.1902/jop.2011.100565

49. Salonen JI, Paunio KU. An intracrevicular washing method for collection of crevicular contents. *Scand J Dent Res*. Oct 1991;99(5):406-12. doi:10.1111/j.1600-0722.1991.tb01048.x
50. Pradeep AR, Raghavendra NM, Prasad MV, Kathariya R, Patel SP, Sharma A. Gingival crevicular fluid and serum visfatin concentration: their relationship in periodontal health and disease. *J Periodontol*. Sep 2011;82(9):1314-9. doi:10.1902/jop.2011.100690
51. Wassall RR, Preshaw PM. Clinical and technical considerations in the analysis of gingival crevicular fluid. *Periodontol 2000*. Feb 2016;70(1):65-79. doi:10.1111/prd.12109
52. Jin L, Yu C, Corbet EF. Granulocyte elastase activity in static and flow gingival crevicular fluid. *J Periodontal Res*. Jun 2003;38(3):303-10. doi:10.1034/j.1600-0765.2003.00606.x
53. Baker HN, Murphy R, Lopez E, Garcia C. Conversion of a capture ELISA to a Luminex xMAP assay using a multiplex antibody screening method. *J Vis Exp*. Jul 6 2012;(65)doi:10.3791/4084
54. Medo M, Aebersold DM, Medova M. ProtRank: bypassing the imputation of missing values in differential expression analysis of proteomic data. *BMC Bioinformatics*. Nov 9 2019;20(1):563. doi:10.1186/s12859-019-3144-3
55. Kong W, Hui HWH, Peng H, Goh WWB. Dealing with missing values in proteomics data. *Proteomics*. Dec 2022;22(23-24):e2200092. doi:10.1002/pmic.202200092
56. Harris L, Fondrie WE, Oh S, Noble WS. Evaluating Proteomics Imputation Methods with Improved Criteria. *J Proteome Res*. Nov 3 2023;22(11):3427-3438. doi:10.1021/acs.jproteome.3c00205
57. Tyanova S, Temu T, Sinitcyn P, et al. The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nat Methods*. Sep 2016;13(9):731-40. doi:10.1038/nmeth.3901
58. Cavalli G, Tengesdal IW, Gresnigt M, et al. The anti-inflammatory cytokine interleukin-37 is an inhibitor of trained immunity. *Cell Rep*. Apr 6 2021;35(1):108955. doi:10.1016/j.celrep.2021.108955
59. Ho JY, Yeo BS, Yang XL, et al. Local and Systemic Expression Profile of IL-10, IL-17, IL-27, IL-35, and IL-37 in Periodontal Diseases: A Cross-sectional Study. *J Contemp Dent Pract*. Jan 1 2021;22(1):73-79.
60. Xie L, Huang Z, Li H, Liu X, Zheng S, Su W. IL-38: A New Player in Inflammatory Autoimmune Disorders. *Biomolecules*. Aug 5 2019;9(8)doi:10.3390/biom9080345
61. Navazesh M. Methods for collecting saliva. *Ann N Y Acad Sci*. Sep 20 1993;694:72-7. doi:10.1111/j.1749-6632.1993.tb18343.x
62. Guru SR, Aghanashini S. Impact of scaling and root planing on salivary and serum plasminogen activator inhibitor-1 expression in patients with periodontitis with and without type 2 diabetes mellitus. *J Periodontol*. Jan 2023;94(1):20-30. doi:10.1002/JPER.22-0129
63. Kim JY, Kim HN. Changes in Inflammatory Cytokines in Saliva after Non-Surgical Periodontal Therapy: A Systematic Review and Meta-Analysis. *Int J Environ Res Public Health*. Dec 29 2020;18(1)doi:10.3390/ijerph18010194
64. Teles FRF, Chandrasekaran G, Martin L, et al. Salivary and serum inflammatory biomarkers during periodontitis progression and after treatment. *J Clin Periodontol*. Aug 5 2024;doi:10.1111/jcpe.14048
65. Isaza-Guzman DM, Cardona-Velez N, Gaviria-Correa DE, Martinez-Pabon MC, Castano-Granada MC, Tobon-Arroyave SI. Association study between salivary levels of interferon (IFN)-

gamma, interleukin (IL)-17, IL-21, and IL-22 with chronic periodontitis. *Arch Oral Biol*. Jan 2015;60(1):91-9. doi:10.1016/j.archoralbio.2014.09.002
66. Medovic MV, Jakovljevic VL, Zivkovic VI, et al. Psoriasis between Autoimmunity and Oxidative Stress: Changes Induced by Different Therapeutic Approaches. *Oxid Med Cell Longev*. 2022;2022:2249834. doi:10.1155/2022/2249834
67. Fonseca-Camarillo G, Furuzawa-Carballeda J, Iturriaga-Goyon E, Yamamoto-Furusho JK. Differential Expression of IL-36 Family Members and IL-38 by Immune and Nonimmune Cells in Patients with Active Inflammatory Bowel Disease. *Biomed Res Int*. 2018;2018:5140691. doi:10.1155/2018/5140691
68. Yang CS, Zhang QX, Deng Y, et al. Increased serum IL-36beta and IL-36gamma levels in patients with neuromyelitis optica spectrum disorders: association with disease activity. *BMC Neurol*. Aug 5 2019;19(1):185. doi:10.1186/s12883-019-1415-2
69. Toraman A, Saglam E, Savran L, Koseoglu S. Evaluation of Salivary Il-38 Levels in Periodontitis: A Cross-Sectional Study. *J Interferon Cytokine Res*. Mar 18 2024;doi:10.1089/jir.2023.0233
70. Cicchese JM, Evans S, Hult C, et al. Dynamic balance of pro- and anti-inflammatory signals controls disease and limits pathology. *Immunol Rev*. Sep 2018;285(1):147-167. doi:10.1111/imr.12671
71. Al-Qahtani AA, Alhamlan FS, Al-Qahtani AA. Pro-Inflammatory and Anti-Inflammatory Interleukins in Infectious Diseases: A Comprehensive Review. *Trop Med Infect Dis*. Jan 4 2024;9(1)doi:10.3390/tropicalmed9010013
72. Cheng R, Wu Z, Li M, Shao M, Hu T. Interleukin-1beta is a potential therapeutic target for periodontitis: a narrative review. *Int J Oral Sci*. Jan 2 2020;12(1):2. doi:10.1038/s41368-019-0068-8
73. Wang Q, Ma L, An C, Wise SG, Bao S. The role of IL-38 in intestinal diseases - its potential as a therapeutic target. *Front Immunol*. 2022;13:1051787. doi:10.3389/fimmu.2022.1051787
74. Theoharides TC, Tsilioni I, Conti P. Mast Cells May Regulate The Anti-Inflammatory Activity of IL-37. *Int J Mol Sci*. Jul 29 2019;20(15)doi:10.3390/ijms20153701
75. Zhang X, Wang Z, Zheng Y, et al. Inhibitors of the NLRP3 inflammasome pathway as promising therapeutic candidates for inflammatory diseases (Review). *Int J Mol Med*. Apr 2023;51(4)doi:10.3892/ijmm.2023.5238
76. Balta MG, Papathanasiou E, Blix IJ, Van Dyke TE. Host Modulation and Treatment of Periodontal Disease. *J Dent Res*. Jul 2021;100(8):798-809. doi:10.1177/0022034521995157
77. Van Dyke TE. Shifting the paradigm from inhibitors of inflammation to resolvers of inflammation in periodontitis. *J Periodontol*. Oct 2020;91 Suppl 1(Suppl 1):S19-S25. doi:10.1002/JPER.20-0088
78. Hasturk H, Kantarci A, Goguet-Surmenian E, et al. Resolvin E1 regulates inflammation at the cellular and tissue level and restores tissue homeostasis in vivo. *J Immunol*. Nov 15 2007;179(10):7021-9. doi:10.4049/jimmunol.179.10.7021
79. Ridker PM. Anticytokine Agents: Targeting Interleukin Signaling Pathways for the Treatment of Atherothrombosis. *Circ Res*. Feb 2019;124(3):437-450. doi:10.1161/CIRCRESAHA.118.313129
80. Pani P, Tsilioni I, McGlennen R, et al. IL-1B(3954) polymorphism and red complex bacteria increase IL-1beta (GCF) levels in periodontitis. *J Periodontal Res*. Jun 2021;56(3):501-511. doi:10.1111/jre.12850

81. Song J, Zhang Y, Zhou C, et al. The dawn of a new Era: mRNA vaccines in colorectal cancer immunotherapy. *Int Immunopharmacol*. May 10 2024;132:112037. doi:10.1016/j.intimp.2024.112037