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Salivary Biomarkers of Periodontal Disease in Smoker and Non-Smoker Periodontitis Patients

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Abstract

Background: Smoking alters the host response, including vascular function, neutrophil/monocyte activities, antibody production, and cytokine and inflammatory mediator release. Interleukin-1β (IL-1β), osteoprotegerin (OPG) and matrix metalloproteinase 8 (MMP8) are involved in three aspect of periodontal disease: inflammation, collagen degradation and bone resorption. Therefore, measurement of salivary concentration of these biomarkers could be used as a reliable indicator of periodontal disease activity. Hence, the objectives of this study were to determine and compare the salivary concentrations of IL-1β, MMP8 and OPG in smokers versus non-smokers with periodontal disease as well as healthy individuals and determine if they correlate with clinical features of periodontal disease**.**

Methods: A total of 87 Participants (30 periodontaly healthy (H), 28 non-smoker individuals with periodontitis (P), 29 smoker individuals with periodontitis (SP)) formed the study group. After periodontal examination including probing depth (PD), clinical attachment level (CAL) and bleeding on probing, saliva samples were collected and the amounts of IL-1β, MMP8 and OPG were quantified using enzyme-linked immunosorbent assays.

Results: Bleeding was found to be statistically significantly ($p = 0.001$) higher in periodontitis and smoker periodontitis groups compared to controls (2.62±0.48,

1.82±0.47, and 0.74±0.66; respectivel),($p = 0.001$). IL-1β and MMP8 levels in saliva were found to be highest in smokers with periodontitis (7.25±7.14, 3494.80±1092.89 respectively), OPG level in saliva were found to be highest in periodontitis (744.79±1617.68) without statistical significance ($p = 0.374$). Weak correlations were found between level of OPG with PD and CAL that was statistically significant ($p = 0.019$ and 0.012; respectively).

Conclusion: Bleeding level is suggested to be affected by smoking habits. Longitudinal, prospective studies will help to verify the observations of the present study. Further research in this direction could reveal reliable markers to forecast the progress of periodontal disease in smoker subjects.

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I. Introduction

a. Background and Rationale

1. Periodontal disease

Periodontal disease is a chronic bacterial infection characterized by persistent inflammation, connective tissue destruction and alveolar bone resorption. [1-3] Periodontal disease is a major cause of tooth loss. [4] The clinical signs of periodontal disease vary depending on the rate and severity of tissue destruction (Figure 1). [1-7] Usually it is a slowly progressive disease but some patients may experience short periods of rapid progression. [8-10] Susceptibility to periodontal disease differs according to an individual's bacterial flora and local and systemic factors. [1]

There is general consensus that chronic periodontitis is initiated and sustained by dental plaque biofilm, but the host's response to bacterial plaque is responsible for most of the tissue destruction. [11-13] The extent and severity of the disease can be increased by several host-modifying factors, including poorly controlled diabetic mellitus, [11] cigarette smoking, [12] and emotional distress. [13] The progression of the disease can only be confirmed by repeated clinical examinations which should show additional connective attachment loss and resorption of alveolar bone. It is generally assumed that the risk for progression is increased if the disease is untreated. Treatment of chronic periodontitis is directed against disruption, removal and control of the subgingival plaque biofilm. [14]

2. Risk Factors

Periodontal disease is considered to have multiple risk factors. Smoking is a well-established risk factor for periodontal disease and is associated with a 2 to 8 fold increase in the risk for periodontal attachment and bone loss, depending on disease severity and cigarette use.[15] The relationship between smoking and periodontal disease has been studied, providing evidence of a positive association between smoking and clinical and radiographic signs of periodontitis, as well as an increased risk of periodontitis in smokers.[15-18]

Smoking not only increases the destruction of periodontal tissue but also makes diagnosis a challenge. A decrease in gingival redness and bleeding on probing was found in smokers with gingivitis when compared to nonsmokers.[19] Smokers with periodontitis have more alveolar bone loss, gingival recession,[20-22] and more tooth loss[23] when compared to nonsmokers with periodontitis. Smoking also has a profound effect on the immune system, resulting in increased number of leukocytes in the systemic circulation, but fewer cells may migrate into the gingival pocket.[24]

The diagnosis of periodontal disease is based traditionally on clinical parameters, which reflect a history of periodontal disease but cannot predict future disease activity.[25] Early diagnosis of ongoing tissue destruction in progressive periodontitis is particularly important in order to prevent further irreversible attachment loss.[25–27]

3. Inflammatory biomarkers

Periodontal disease begins with inflammation of the gingival apparatus and periodontal tissues including the gingiva, periodontal ligament and alveolar bone, in response to bacterial plaque accumulation.[26] The persistent presence of the multi- species bacterial biofilm leads to chronic inflammation and an abundance of inflammatory molecules in oral fluids.[27] To date, several studies have detected one or more inflammatory markers at higher concentrations in patients who have periodontal disease compared to healthy controls. Several of these inflammatory molecules also serve as bone resorptive factors.[27]

Interleukin-1β (IL-1β) is a key pro-inflammatory cytokine that is released after infection, injury or antigenic challenge.[28] It is widely accepted as a biomarker of systemic inflammation.[28] IL-1β is principally produced by activated macrophages, but it is also secreted by a wide variety of epithelial, lymphoid, and vascular tissues.[28, 29] IL-1β is considered the prototypical 'multi-functional' cytokine, affecting nearly all cell types, either alone or in combination with other cytokines.[28] It serves an important role in promoting the production of other pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), IL-6, and IL-1α, as well as inducing the acute phase response[28, 30, 31].

IL-1β is present in the saliva of both healthy and diseased individuals.[32] The sources of IL-1β in the oral cavity include macrophages, monocytes, fibroblasts, and mucosal epithelial cells[32, 33]. IL-1β has also been found to be synthesized and released from acinar and ductal cells in mouse salivary glands.[34, 35, 36] Human gingival crevicular fluid (GCF), which may be present as component of whole saliva, also contains IL-1β.[37] Levels of IL-

1β in saliva and CGF have been studied in relation to gingival and periodontal disease.[36, 38-42]

Matrix metalloproteinase (MMPs) are a family of proteolytic enzymes secreted by neutrophils, macrophages, epithelial cells, fibroblasts, osteoblasts and osteoclasts.[43] The primary function of MMPs is to degrade the extracellular matrix components (collagen, laminin, fibronectin), and therefore they are important in tissue remodeling.[43] Although periodontal pathogens produce MMPs, endogenous MMPs are primarily responsible for tissue destruction associated with periodontal disease.[43, 44]

MMP-8 is a key enzyme in extracellular collagen matrix degradation[45,46] secreted predominantly by polymorphonuclear leukocytes during acute stages of periodontal disease, leading to periodontal tissue destruction as a result of the degradation of gingival and periodontal ligament collagen.[47]

Osteoprotegerin (OPG) is a glycoprotein that acts as an osteoblast secreted decoy receptor.[48] It inhibits osteoclast differentiation and activity competitively by preventing osteoclast differentiation factor or the receptor activator of NF-κB ligand (RANKL) from binding to osteoclast precursors. OPG promotes the formation of bone-resorbing osteoclasts.[48,49] By binding to RANKL, OPG prevents its further interaction with RANK, and subsequently, all the down-stream molecular events that lead to osteoclast differentiation and bone resorption.[50] The production of RANKL and OPG by various cell types is controlled by systemic and local stimuli, including hormones, inflammatory mediators and bacterial products.[50, 51]

Increased RANKL or decreased OPG local expression can cause bone resorption at various sites in the human skeleton.[52] Conversely, decreased RANKL or increased OPG expression could result in enhanced bone formation, leading to osteopetrotic conditions.[53] Among various biomarkers of bone destruction, the investigation of RANKL and OPG may deliver reliable information on the state of periodontal health status, but may not be able to predict future activity.[53]

4. Saliva as a diagnostic fluid

Saliva is appealing for use as a diagnostic fluid for oral diseases because it is rapid, easy and non-invasive to collect, and generally readily abundant[36, 53-55] The recent cataloguing of the salivary proteome has availed considerable information that is potentially important for diagnostic applications.[56-58] The clinical utility of this information is pertinent to the type of saliva being analyzed, which can be either glandular- specific saliva or whole saliva. There are three major salivary glands (parotid, submandibular, and sublingual) that introduce saliva to the oral cavity. Saliva from these glands provide different mixtures of serous- and mucinous-derived fluid, and can be used for the detection of gland-specific pathology. [59] Whole saliva, by contrast, is composed of a mixture of oral fluids from the major salivary (submandibular 65%, parotid 23% and sublingual 4%) and minor salivary glands (8%).[59] It also contains constituents of non salivary origin, including GCF, serum transudate from the mucosa and sites of inflammation, epithelial and immune cells, food debris and many microbes.[59,60]

There is a shortage of studies using saliva as a diagnostic tool for smoker periodontitis patients to overcome the masking effect of smoking on the clinical features of periodontal disease. Therefore, the aim of this study was to determine if there was a correlation between clinical features of periodontal disease including probing pocket depth (PPD), bleeding on probing (BOP) and clinical attachment loss (CAL) and the levels of three salivary biomarkers interleukin-1β (IL-1β), osteoprotegerin (OPG) and matrix metalloproteinase 8 (MMP8) in smoker and non-smoker periodontitis patients. Another aim is to determine the role of cigarette smoking in masking the clinical features of periodontal disease and the validity of the salivary biomarkers (IL-1β, OPG, and MMP8) as a prognostic tool in periodontitis treatment.

b. Hypothesis

There will be a positive correlation between the clinical features of periodontal disease (PPD, BOP, and CAL) and the levels of the salivary biomarkers (IL-1β, OPG, and MMP-8) in periodontitis patients. Cigarette smoking will alter this correlation.

II. Material and Methods

A. Experimental design

This study is a single center cross-sectional study, to determine if the levels of IL-1β, MMP-8 and OPG in saliva correlate with the clinical features of periodontal disease and cigarette use.

B. Study Characteristics

This study included 87 subjects from among those who are seeking periodontal treatment at the postgraduate and undergraduate clinics at Tufts University School of Dental Medicine (TUSDM). Subjects fulfilling the inclusion and exclusion criteria who wish to participate in the study were introduced to the research plan. Based on periodontal examination and diagnosis, the subjects were classified to one of three groups:

Group 1 - Non-smokers with healthy periodontium (H).

Group 2 - Non-smokers with periodontitis (P).

Group 3 – Smokers with periodontitis (SP)

C. Inclusion criteria

General Inclusion criteria

1. Must be at least 18 years of age.
2. Display no evidence of acute periodontal infection (e.g., abscess).
3. Have at least 6 teeth in both maxillary and mandibular arches.

- **Non-smoker healthy periodontium group inclusion criteria**

1. Non-smoker
2. Has PD of no greater than 3 mm in all of their periodontal sites.
3. Shows less than 10 percent of BOP in their periodontal sites.

4. Has no evidence of radiographic bone loss when viewed on posterior bitewing films. [61]

- **Non-smoker periodontitis group inclusion criteria**

1. Existing generalized moderate to severe chronic periodontitis. [61]
2. Greater than 2 millimeters radiographic bone loss in patient's posterior bitewing films.

- **Smoker periodontitis group inclusion criteria**

1. Has smoked more than 10 cigarettes per day for at least in the past 6 months.
2. Existing generalized moderate to severe chronic periodontitis.[61]
3. Greater than 2 millimeters radiographic bone loss in patient's posterior bitewing films.

D. Exclusion criteria

1. Have any known systemic disease or are currently on medication with a direct effect on periodontal tissue (e.g., uncontrolled hypertension, uncontrolled or poorly controlled diabetes, HIV/AIDS (self-reported), history of or currently undergoing head and neck radiation therapy).
2. Be pregnant or lactating (self- reported).
3. Heavy alcohol consumption affecting periodontal health.
4. Have liver or kidney dysfunction, inflammatory bowel disease, granulomatous disease or immunosuppression.
5. Are undergoing or have undergone organ transplantation or cancer therapy.

6. Have used antibiotics or immunosuppressant medication within the last three months.
7. In need of antibiotics for infective endocarditis prophylaxis during dental procedures.
8. Have symptoms of acute illness (such as fever, sore throat and diarrhea)
9. Detection of an oral mucosal inflammatory condition (e.g., aphthae, lichen planus, leukoplakia or oral cancer).

E. Study Procedures

The risks and benefits of participation in the study were explained to the subjects selected and they were asked to read the consent form, questions concerning the protocol were answered. subjects certified their willingness to participate in the study by signing and dating the Institutional Review Board (IRB) approved informed consent form, a copy of which was provided to the patient.

Visit 1: Screening and periodontal examination.

1. Study subjects were recruited during a regularly scheduled visit. Informed consent was obtained by the principal investigator or a co-investigator.
2. The study subjects were instructed to read the informed consent form (ICF), given ample time to have any questions answered, and then instructed to sign the ICF.
3. Study subjects were given a copy of the ICF.
4. Study subjects were asked to complete demographic information and a medical history.

5. Inclusion/exclusion criteria were evaluated.
6. Clinical features of periodontal disease were determined in all enrolled patients, which included PPD, BOP and CAL.

a. **Probing pocket depth (PPD):** Was measured in millimeters to the nearest millimeter with manual periodontal probe at 6 sites (mesio-buccal, mid- buccal, disto-buccal, mesio-lingual, mid-lingual and disto-lingual) around each tooth.

b. **Clinical attachment level (CAL):** Was measured in millimeters from the cementoenamel junction to the free gingival margin on the facial and lingual side of each tooth.

c. **Bleeding on probing using bleeding time index (BTI):** Was recorded at 6 sites (mesio-buccal, mid- buccal, disto-buccal, mesio-lingual, mid-lingual and disto-lingual) around each tooth, time for bleeding to occur was recorded using BTI [(62)], at the same time of PD recording;(Table 1).

Visit 2: Saliva sample collection:

Study subjects were instructed not to eat, smoke or drink for at least one hour prior to the collection of saliva. Furthermore, all collections were performed at a fixed time of the day (3 hours window) to minimize fluctuations related to the circadian rhythm in salivary secretion. The patients were seated and the floor of the mouth was isolated with a sterile gauze pad. Stimulated (using lemon juice) or unstimulated saliva was collected from the

submandibular glands (Wharton's duct) using a suction device (Figure 2). Saliva samples, approximately 2 mL, were transferred on ice and kept at -80°C until analysis [63]

F. Laboratory tests:

Each sample was transferred to a clean 1 mL microfuge tube and centrifuged for 20 min at 12,000 rpm in a bench top refrigerated centrifuge.

Enzyme linked immunosorbent assays (ELISA) were used to determine the concentration of the salivary biomarkers IL-1β, MMP-8 and OPG for each study subject.

I. IL-1β, MMP8 and OPG ELISA:

The manufacturer guidelines were followed for each assay (R&D Systems Inc. Minneapolis, MN, USA). The optical density of each sample was determined using a microplate reader. The salivary concentrations of IL-1β, OPG, RANKL and MMP8 were then determined by comparing the average absorbance reading of each sample with the concentration in the assay standard curve.

III. Sample Size Calculation

The sample size calculation was performed using nQuery advisor (version 7.0). Based on data from the literature, inclusion of 25 study subjects per group (75 subjects) is adequate to achieve type I error, alpha = 0.05 and power of 80%.

IV. Statistical Analysis

Statistical analysis of the data was performed using SPSS statistical program (version 15.0; SPSS Inc., Chicago, IL, USA). Non-parametric test was applied in data analysis. Demographic variables (age, gender and race) were compared between the three groups using chi-square test Statistical significant was determined at the 0.05 level (Table 1).

Medians of teeth number, bleeding, recession, probing depth, clinical attachment level, IL-1β, OPG and MMP8 levels were compared using Kruskal-Wallis H-test (Tables 2, 3). Study subjects were compared according to gender using Kruskal-Wallis H-test test in Table 4 and 5. Further analyses was conducted to evaluate the difference between groups using Mann-Whitney U, statistical significance was determined at the .016 level with correction for multiple comparisons.

Spearman's correlation was performed to investigate the relationship between the salivary biomarkers and clinical parameters (Tables 6, 7). Statistical significance was determined at the 0.05 level, unless otherwise noted.

Mutable regression was tested to determine if is possible to predict the clinical condition by using the level of biomarkers in the saliva sample (dependent variable) (Tables10, 11, and 12).

V. Results:

Demographic data

This study enrolled 87 subjects with a median age of 47.0[13.80 years and were assigned to one of three groups on the basis of periodontal health and smoking habit: non-smokers with healthy periodontium , non-smokers with periodontitis and smokers with periodontitis. All subjects were otherwise systemically healthy. The healthy group included 30 subjects who had no evidence of any ongoing periodontal disease. Periodontitis group included 28 subjects that had recently been diagnosed with periodontitis. The smoker periodontitis group included 29 subjects who had recently been diagnosed with periodontitis. Table 3 summarizes the demographic data of the study subjects: 63.2% of the subject included in this study were male and 36.8% female; 60.9% were white, 18.4% African American, 12.6% Hispanic and 8% Asian.

Clinical Evaluation

The Median [IQR] of the number of teeth, bleeding, probing depth, clinical attachment level and recession were determined for all three groups and are summarized in Table 4. The mean number of teeth was lower in the periodontitis (24.61[3.47]) and smoker

periodontitis group (24.55[4.17]) when compared to the healthy group (26.77[1.76]) and this difference was statistically significant ($p<0.015$). Bleeding in periodontitis group (2.62[0.48]) was higher than in smoker periodontitis group (1.82[0.47]) and the healthy group (0.74[0.66]), the differences were statistically significant ($p<0.001$). The mean probing depth was statistically significantly ($p<0.001$) higher in the periodontitis and smoker periodontitis group (3.32[0.27] and 3.42[0.88], respectively) compared to the healthy group (2.27[0.38]). Clinical attachment level in the periodontitis group was 4.11[0.6] and smoker periodontitis group was 4.3[1.24], and both were higher than in the healthy group (2.58[0.74]) with the difference being statistically significant ($p<0.001$). The mean recession was statistically significantly ($p<0.001$) higher in the periodontitis and smoker periodontitis group (0.80[0.46] and 0.89[0.54], respectively) compared to the healthy group (0.32[0.46]).

Salivary levels of IL-1β, MMP-8, OPG and RANKL.

The concentration of IL-1β, MMP-8 OPG and RANKL in saliva was determined using commercially available ELISAs. Table 5 shows that the mean salivary levels of IL-1β, MMP-8 and OPG were higher in the smoker periodontitis subjects (7.25[7.14], 3494[1092.89] and 509.26[725]; respectively) than the healthy (6.11[10.66], 1780.65[2241.61] and 261.59 [358.04]) and periodontitis subjects (6.07[5.99], 2304.69[2593.93] and 744.79 [1617.68]). Salivary OPG levels were elevated in periodontitis subjects (744.79[1617.68]) compared to 261.59[358.04] in healthy subjects and 509.26[725] in smoker periodontitis subjects. The differences in all biomarkers concentration between groups were not statistically significant. RANKL concentration in

our sample was low and under the level of detection.

When the subjects were divided according to gender, in Table 6 and 7, the groups showed similar results without any diversity. The differences in the clinical parameters and biomarkers concentration between groups were not statistically significant.

Correlation between salivary biomarkers and clinical parameters

The correlation between the concentration of biomarkers in saliva and clinical parameters was evaluated in the study subjects (Table 8). Only a moderate correlation between probing depth and clinical attachment level with OPG was statistically significant. In contrast, IL-1β, MMP-8 concentration in saliva showed a weak correlation with the clinical parameters with no statistical significance in all the results.

The correlation between the biomarkers concentration with clinical parameters was evaluated for each group separately (Table 9 and Figures 8-19). In the healthy group, bleeding, probing depth and clinical attachment levels were positively correlated with the level of MMP8 with statistically significant results. On the other hand a moderate positive correlation was found between clinical attachment level and OPG level in saliva and this correlation was found to be statistically significant. The correlation between clinical parameter and biomarkers levels in saliva was found to be positive but weak in periodontitis group and smoker periodontitis group with no statistically significant outcomes.

VI. Discussion

in the present study, we analyzed the salivary levels of IL-1β, MMP and OPG, as well as the periodontal condition in 87 systemically healthy subjects; 57 of whom s had been diagnosed with chronic periodontitis (29 smokers and 28 non-smokers). As a study specimen, saliva has the advantages of being easily and non-invasively collected.

Cigarette smoking is a significant risk factor for the initiation and progression of periodontal disease.[64] It has been demonstrated that patients who smoke have more potential to develop periodontal disease compared to non-smokers. [66] The inhibition of the inflammatory, destructive effect of cigarette smoke on periodontal tissue, coupled with the possible vasoconstrictive effect of tobacco products, might explain the attachment loss, bone loss and tooth loss in former smokers but, paradoxically, there are fewer signs of inflammation and greater reduction of bleeding upon probing.[67, 68] The present study showed a statistically significant difference in teeth number between smoking periodontitis subjects and healthy subjects.

In the present study, bleeding was found to be a critical sign of inflammation in periodontal disease; being significantly lower in smoking patients compared to non-smoking periodontitis patients. On the other hand, probing depth, clinical attachment level and recession were similar in both smoking and non-smoking periodontitis subjects. This might reflect the masking effect of smoking on periodontal tissue where the same destruction and breakdown of tissue by periodontal disease is observed without any inflammatory signs of disease progression. This observation is supporting previous studies where a great reduction in bleeding had been reported in smoking patients with the existence of periodontal disease. [68, 69]

Several studies have shown that smoking impairs various aspects of the innate and

adaptive immune responses, including altered neutrophil function, antibody production, altered fibroblast activity and inflammatory mediators production.[70, 71] Smoking affects many aspects of the host's immune response; therefore, it is possible that these effects on the immune system could be primary contributing factors of smoking to the pathogenicity of periodontal disease.[72] Cytokines, such as IL-1, IL-6 and IL-8, are considered to be involved in the host response to periodontal disease as mediators of tissue breakdown.[73] Increased levels of IL-1β in GCF have been associated with gingival inflammation, severe periodontitis and periodontal disease progression.[74] Although the detection of IL-1β in this study was consistent with previous studies on smoking periodontitis subjects compared to healthy subjects, this difference was not statistically significant.

MMPs are key enzymes, derived predominantly from polymorphonuclear leukocytes during the acute stage of periodontal disease and are involved in degradation of extracellular collagen matrix.[45, 46] Elevated levels of MMPs have been detected in the GCF of periodontitis patients and less frequently in saliva. We investigated the changes in MMP8 levels in periodontitis and smoking periodontitis patients, and found that the levels of MMP8 were elevated in both the periodontitis subjects and smoking periodontitis subjects with no statistical significance. On the other hand, this increase in the saliva level of MMP8 showed a weak correlation with bleeding, probing depth and clinical attachment level.

OPG is a member of the TNF receptor family that is expressed by osteoblasts. It inhibits bone resorption by high affinity binding to RANKL, preventing it from coupling with the RANK receptor.[75] It has been reported that a significantly increased concentration of RANKL and degrees of concentration of the RANKL decoy receptor,

OPG, occur in the GCF of patients with periodontitis.[76] In addition, the RANKL/OPG ratio in the GCF of periodontitis patients has been shown to be higher than in patients without periodontitis.[77] Although the results of the present study did not show any significant differences in the OPG level between the three groups, it was somewhat higher in periodontitis subjects (744.79[1617.68]) compared to healthy (261.59[358.04]) and smoking periodontitis subjects (509.26[725]). This elevation in OPG level does not necessarily indicate a lower RANKL/OPG ratio, since the RANKL level was not investigated in this study. One possible explanation for the elevation in OPG level is that OPG is operating in full capacity yet can overcome the increased number of osteoclasts during periodontal disease, and this will result in more bone resorption.

A good periodontal marker should function regardless of the subject's smoking behavior or other modifications, like anti-microbial medications. [64] A likely limitation of the present study may be the imperfect gender distribution between the groups. Age also could be a confounding factor in interpretation of the present data. Changes in the flow rate could have a significant impact on the concentration of analyzed saliva and the effect of saliva stimulation on the composition of collected samples.

VII. Conclusion

In the present study, we evaluated the correlation between three salivary biomarkers linked with inflammation, collagen degradation and bone turn over. It could be concluded from this study result that bleeding level are suggested to be affected by smoking habit. However, the levels of the three biomarkers were not different between the study groups warranting further studies with a larger population.

VIII. Clinical Implication:

Changes in the levels of salivary biomarkers could have significance in the diagnosis, treatment and progress of periodontal disease.

IX. Future studies:

More longitudinal, prospective studies will help to verify the observation of the present study. These biomarkers could be evaluated before and after receiving periodontal treatment. Further research in this direction could reveal reliable markers to forecast the progress of periodontal disease in smoker subjects.

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Table 1.Bleeding time index

Value	Description
0	No bleeding within 15 seconds of second probing (i.e. 30 seconds total time).
1	Bleeding within 6 to 15 seconds of second probing
2	Bleeding within 11 to 15 of seconds of first probing or 5 seconds after second probing.
3	Bleeding within 10 seconds after initial probing.
4	Spontaneous bleeding.

Table 2. Subject timeline

Appointment	Visit 1	Visit 2
Procedures	Screening	Saliva Sample Collection
Consent Form	X	
Demographics	X	
Medical/Dental History	X	
Inclusion/Exclusion Criteria	X	
Oral Mucosal Tissue Examination	X	X
Periodontal examination	X	
Saliva collection		X
Subject Accountability	X	X
Stipend		X

Table 3. Subject's characteristics of study groups.

Variable	Total N=87	H N=30	P N=28	PS N=29	P-Value
Age	47.03[13.80]	39.400[13.23]	54.64[13.03]	47.59[10.92]	0.001
Gender					0.546
Male	55 [63.2%]	18 [60%]	20 [71.4%]	17 [58.6%]	
Female	32 [36.8%]	12 [40%]	8 [28.6%]	12 [41.4%]	
Race					0.245
White	53 [60.9%]	22 [73.3%]	15 [53.6%]	16 [55.2%]	
African American	16 [18.4%]	2 [6.7%]	7 [25.0%]	7 [24.1%]	
Hispanic	11 [12.6%]	1 [3.3%]	4 [14.3%]	2 [6.9%]	
Asian	7[8.0%]	5 [16.7%]	2 [7.1%]	4 [13.8%]	

Healthy (H), Periodontitis (P), and Smoker Periodontitis (PS). Data represent median [IQR] and number of subject [%].

Table 4. Study population by clinical parameter.

Variable	All Groups N=87	H N=30	P N=28	PS N=29	P value
Number of teeth	25.33[3.40]	26.77[1.76]	24.61[3.47]	24.55[4.17]	0.015
Bleeding	1.71[0.95]	0.74[0.66]	2.62[0.48]	1.82[0.47]	<0.001
Recession (mm)	0.67[0.55]	0.32[0.46]	0.80[0.46]	0.89[0.54]	<0.001
Probing depth (mm)	2.99[0.78]	2.27[0.38]	3.32[0.27]	3.42[0.88]	<0.001
Clinical attachment level (mm)	3.65[1.19]	2.58[0.74]	4.12[0.61]	4.31[1.24]	<0.001

Kruskal-Wallis Test was applied in comparison between Healthy (H), Periodontitis (P), and Smoker Periodontitis (PS) groups. Data represent median [IQR]. p value <0.05.

Table 5. ELSA results from saliva samples of the study group.

Variable	All groups	H	P	PS	P value
	N=87	N=30	N=28	N=29	
IL-1β (pg/ml)	6.48 [8.16]	6.11 [10.66]	6.07 [5.99]	7.25 [7.14]	0.185
OPG (pg/ml)	499.66 [1036.97]	261.59 [358.04]	744.79 [1617.68]	509.26 [725]	0.374
MMP8 (pg/ml)	2520.69 [6574.79]	1780.65 [2241.61]	2304.69 [2593.93]	3494.80 [1092.89]	0.438

Kruskal-Wallis Test was applied in comparison between Healthy (H), Periodontitis (P), Smoker Periodontitis (PS). Data represent median [IQR]. p-values < 0.05 are considered statistically significant.

Table 6. Study population salivary by clinical parameters.

Variable	H/Male N=18	H/ Female N=12	P value	P/Male N=20	P/Female N=8	P value	PS/Male N=17	PS/ Female N=12	P value
Number of teeth	26.83 [1.89]	26.67 [1.61]	0.661	23.90 [3.73]	26.38 [1.92]	0.135	24.65 [4.69]	24.42 [3.50]	0.508
Bleeding	0.90 [0.76]	0.48 [0.34]	0.075	2.65 [0.40]	2.55 [0.65]	0.666	1.74 [0.53]	1.94 [0.38]	0.464
Recession (mm)	0.33 [0.48]	0.30 [0.44]	1.000	0.83 [0.43]	0.74 [0.57]	0.374	0.87 [0.60]	0.91 [0.45]	0.706
PD (mm)	2.33 [0.43]	2.17 [0.29]	0.204	3.28 [0.26]	3.42 [0.31]	0.253	3.42 [0.87]	3.41 [0.94]	0.550
CAL (mm)	2.65 [0.85]	2.47 [0.54]	0.866	4.10 [0.53]	4.14 [0.81]	0.666	4.29 [1.30]	4.31 [1.20]	1.000

Kruskal-Wallis Test was applied in comparison between groups according to gender. Data represent median [IQR].

Healthy (H), Periodontitis (P), Smoker Periodontitis (PS), Probing depth (PD), Clinical attachment loss (CAL). p-values < 0.05 are considered statistically significant.

Table 7. ELSA results from saliva samples of the study group.

Variable	H/ Male N=18	H/ Female N=12	P value	P/ Male N=20	P/ Female N=8	P value	PS/ Male N=17	PS/ Female N=12	P value
IL-1β	6.78 [13.47]	5.09 [4.18]	0.220	5.08 [3.29]	8.55 [9.98]	0.919	7.12 [7.30]	7.44 [7.23]	0.723
OPG	183.95 [191.62]	378.04 [506.51]	0.310	745.29 [1869.76]	743.54 [777.51]	0.373	509.18 [667.02]	509.38 [831.15]	0.723
MMP8	1693.82 [2016.07]	1910.90 [2633.42]	0.641	1906.68 [1956.13]	3299.71 [3739.30]	0.360	3256.56 [14158.74]	3832.30 [14158.74]	0.092

Kruskal-Wallis Test was applied in comparison between groups according to gender. Data represent median [IQR].

Healthy (H), Periodontitis (P), Smoker Periodontitis (PS). p-values < 0.05 are considered statistically significant.

Table 8. Correlation between salivary biomarkers and clinical parameters in all study subjects.

Variable	IL-1β		OPG		MMP8	
	r	P value	r	P value	r	P value
Bleeding	0.075	0.490	0.154	0.155	0.202	0.061
Recession	0.091	0.404	0.207	0.054	0.199	0.065
PD	0.136	0.209	0.252	0.019	0.155	0.152
CAL	0.129	0.235	0.267	0.012	0.202	0.060

Spearman's correlation Test was used to determine the correlation between the biomarkers with the clinical parameters. p-values < 0.05 are considered statistically significant.

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Table 9. Correlation between salivary biomarkers and clinical parameters in study groups.

Variable	Group	IL-1β		OPG		MMP8	
		r	P value	r	P value	r	P value
Bleeding	Healthy	0.095	0.618	0.262	0.163	0.668	0.000
	Periodontitis	-0.270	0.165	0.201	0.305	-0.253	0.194
	Periodontitis with smoking	-.0043	0.823	-0.134	0.487	-0.005	0.981
Recession	Healthy	-0.057	0.764	0.153	0.420	0.151	0.426
	Periodontitis	-0.118	0.549	0.019	0.924	0.148	0.453
	Periodontitis with smoking	0.091	0.640	0.172	0.372	0.014	0.943
PD	Healthy	-0.070	0.712	0.243	0.195	0.361	0.050
	Periodontitis	-0.118	0.549	0.295	0.128	-0.184	0.348
	Periodontitis with smoking	0.186	0.335	0.151	0.435	0.155	0.422
CAL	Healthy	-0.057	0.766	0.368*	0.045	0.418	0.022
	Periodontitis	-0.203	0.301	0.210	0.282	0.049	0.803
	Periodontitis with smoking	0.149	0.441	0.196	0.309	0.082	0.673

Spearman's correlation Test was used to determine the correlation between the biomarkers with the clinical parameters.
p-values < 0.05 are considered statistically significant.

Table 10. Multiple regression. Using the biomarkers level to help predicts the clinical condition.

Variable	Unstandardized Coefficients		Beta	P. Value
	B	Standard Error		
Clinical attachment level	36.46	30.92	1.47	0.242
Probing depth	-28.54	25.98	-0.87	0.275
Recession	-14.14	12.52	-0.69	0.262
Bleeding	-0.52	0.49	-0.21	0.294

The dependent variable is IL-1β. The predictors are recession, bleeding, probing depth and clinical attachment level. p-values < 0.05 are considered statistically significant.

Table 11. Multiple regression. Using the biomarkers level to help predicts the clinical condition.

Variable	Unstandardized Coefficients		Beta	P. Value
	B	Standard Error		
Clinical attachment level	3060.96	3885.41	0.97	0.433
Probing depth	-2785.79	3263.68	-0.67	0.396
Recession	-834.17	1573.52	-0.32	0.597
Bleeding	11.97	61.68	0.04	0.847

The dependent variable is OPG. The predictors recession, bleeding, probing depth and clinical attachment level. p-values < 0.05 are considered statistically significant.

Table 12. Multiple regression. Using the biomarkers level to help predicts the clinical condition.

Variable	Unstandardized Coefficients		Beta	P. Value
	B	Standard Error		
Clinical attachment level	15367.93	25007.86	0.77	0.541
Probing depth	-10207.57	21006.14	-0.39	0.628
Recession	-4735.22	10127.73	-0.29	0.641
Bleeding	-260.93	397.01	-0.13	0.513

The dependent variable is MMP8. The predictors are recession, bleeding, probing depth and clinical attachment level. p-values < 0.05 are considered statistically significant.

Figure 1. Progress of Periodontal disease.

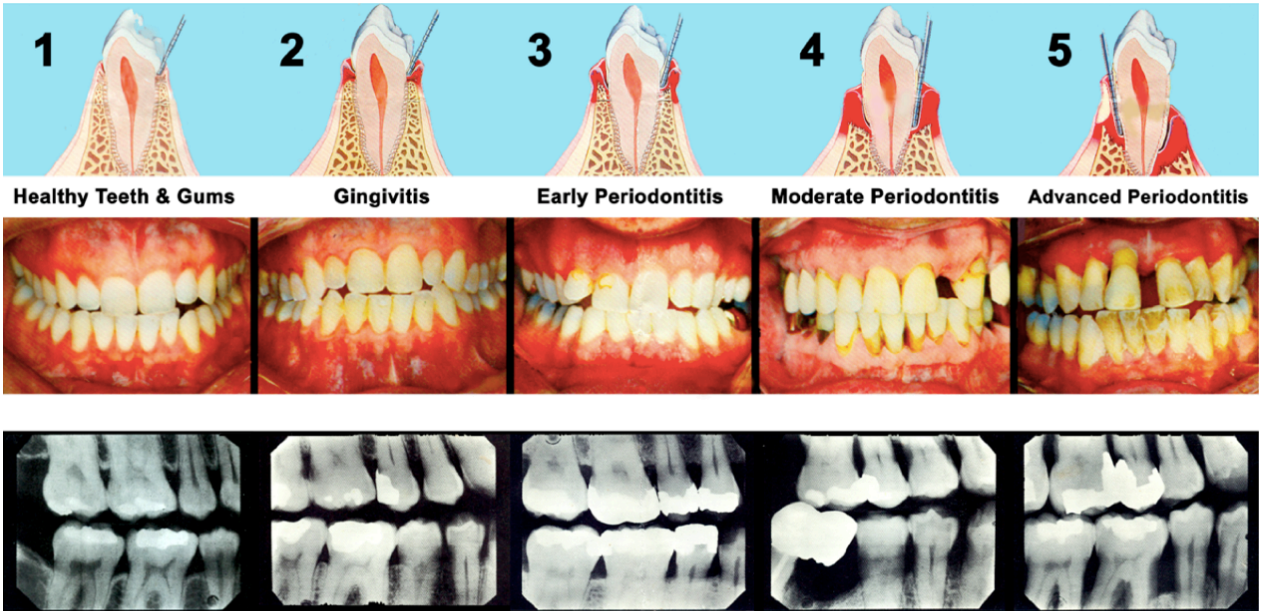
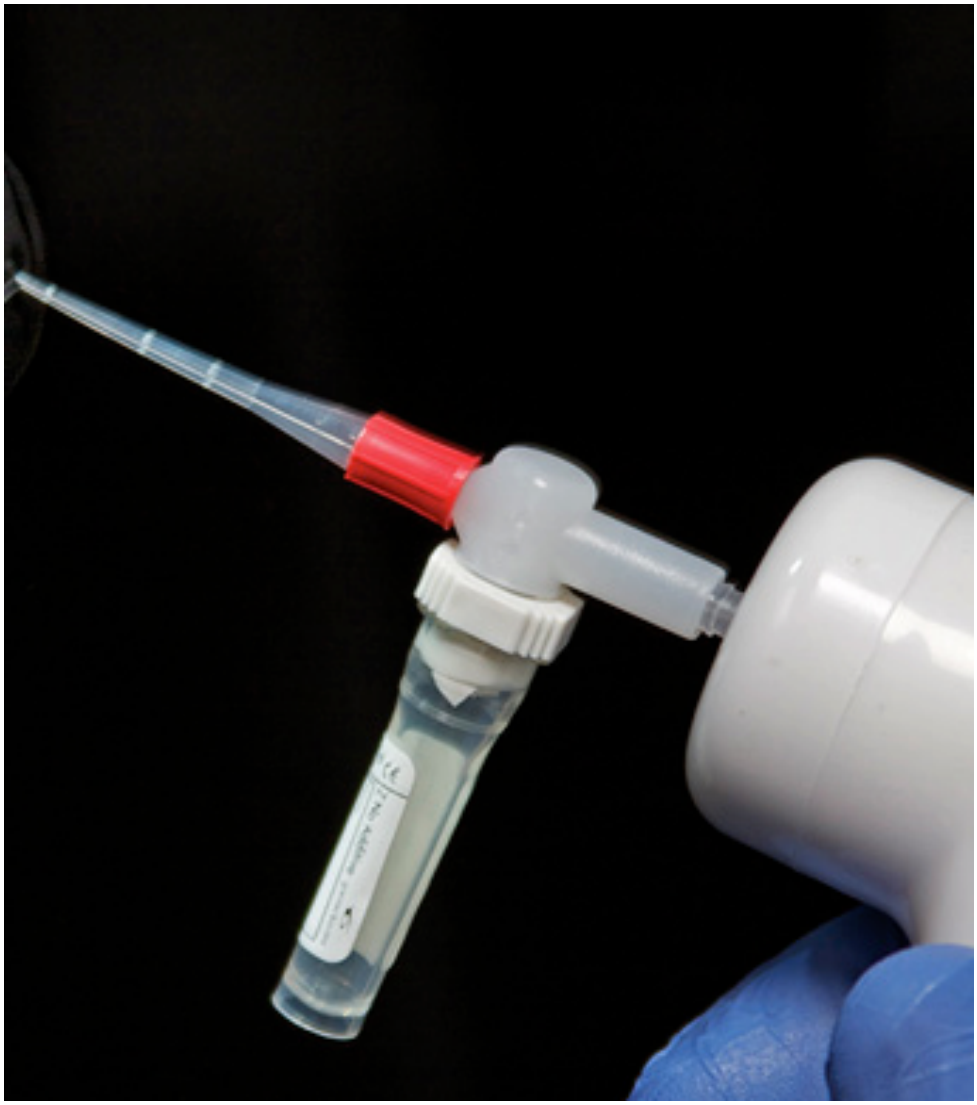


Figure 2. ETD suction device used in saliva collection.



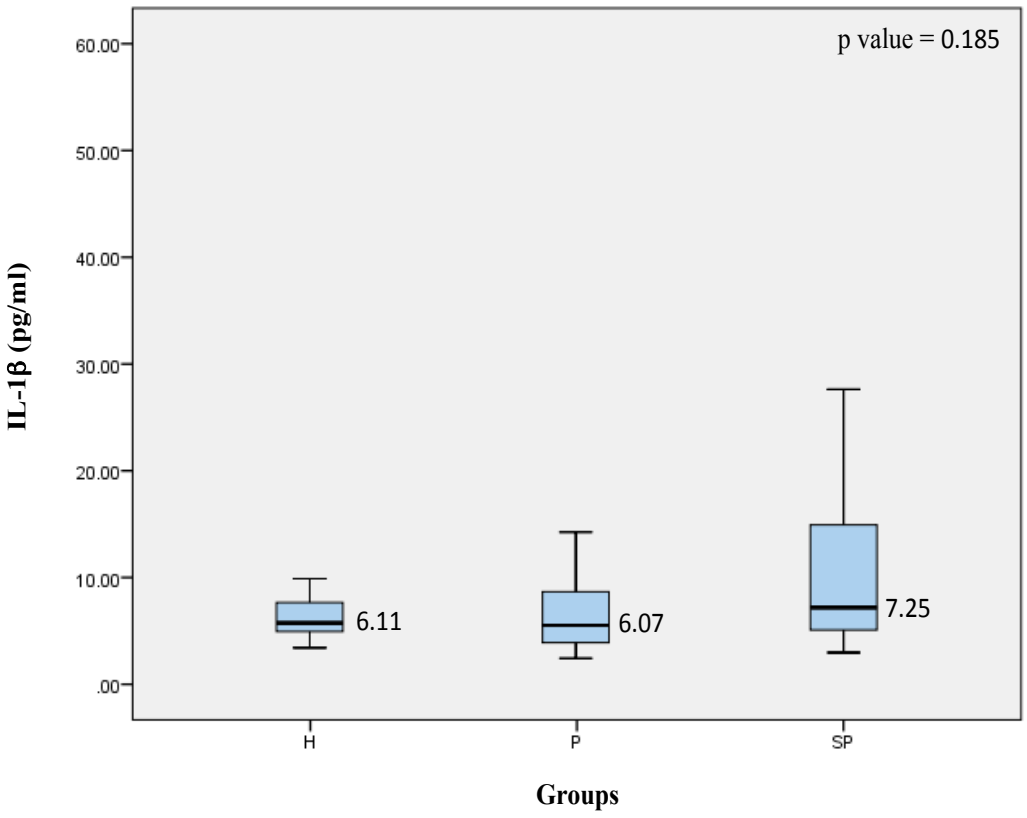


Figure 4: Saliva level of IL-1β in periodontal healthy group (H), periodontitis group (P) and smoker periodontitis group (SP). Data expressed as median.

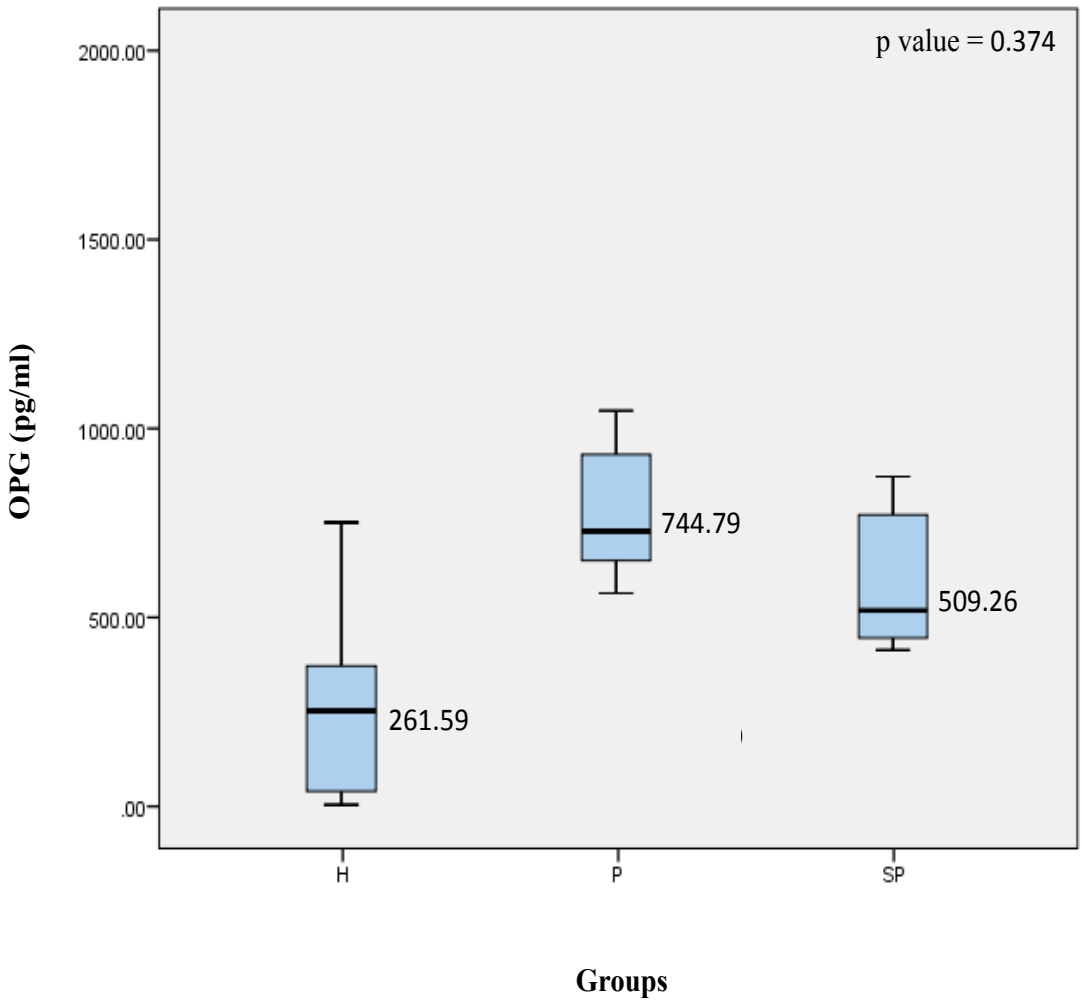


Figure 5: Saliva level of OPG in periodontal healthy group (H) periodontitis group (P) and smoker periodontitis group (SP). Data expressed as median.

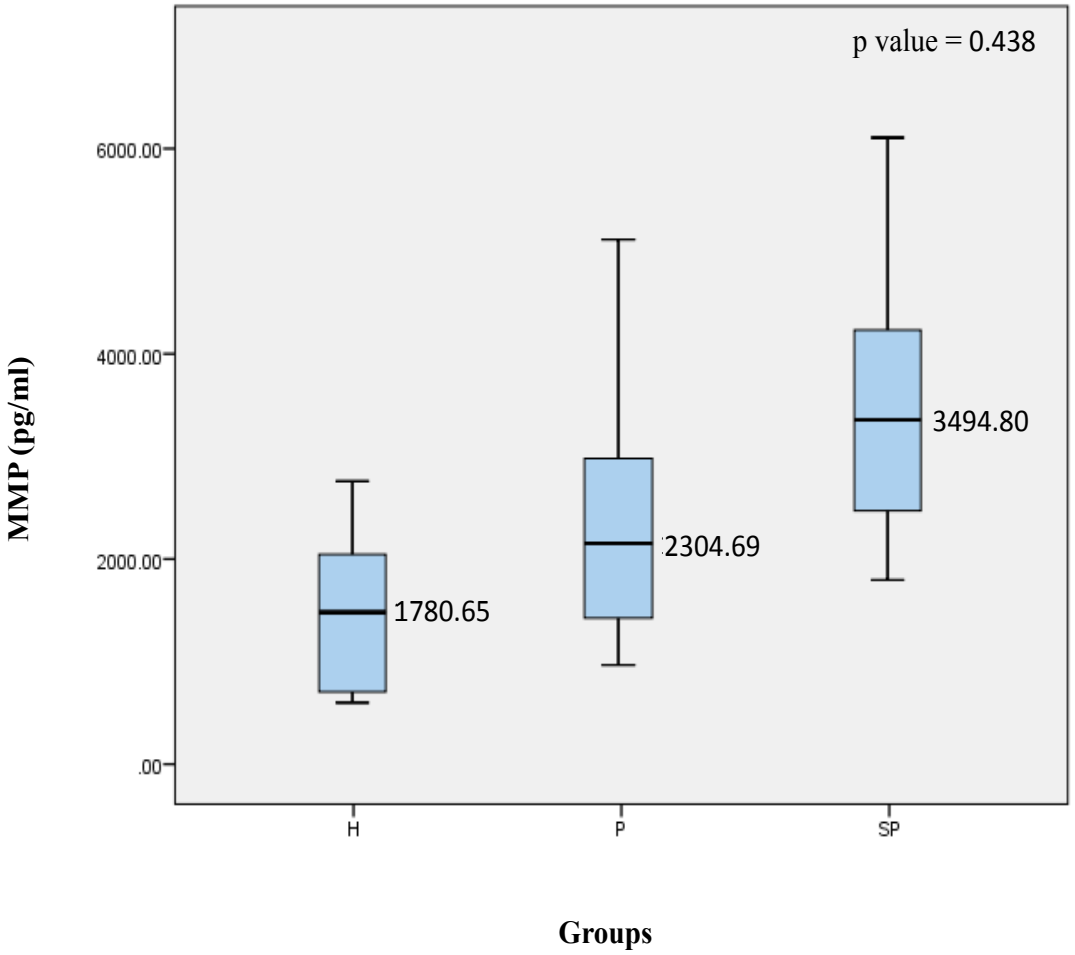


Figure 6: Saliva level of MMP 8 in periodontal healthy group (H), periodontitis group (P) and smoker periodontitis group (SP). Data expressed as median.

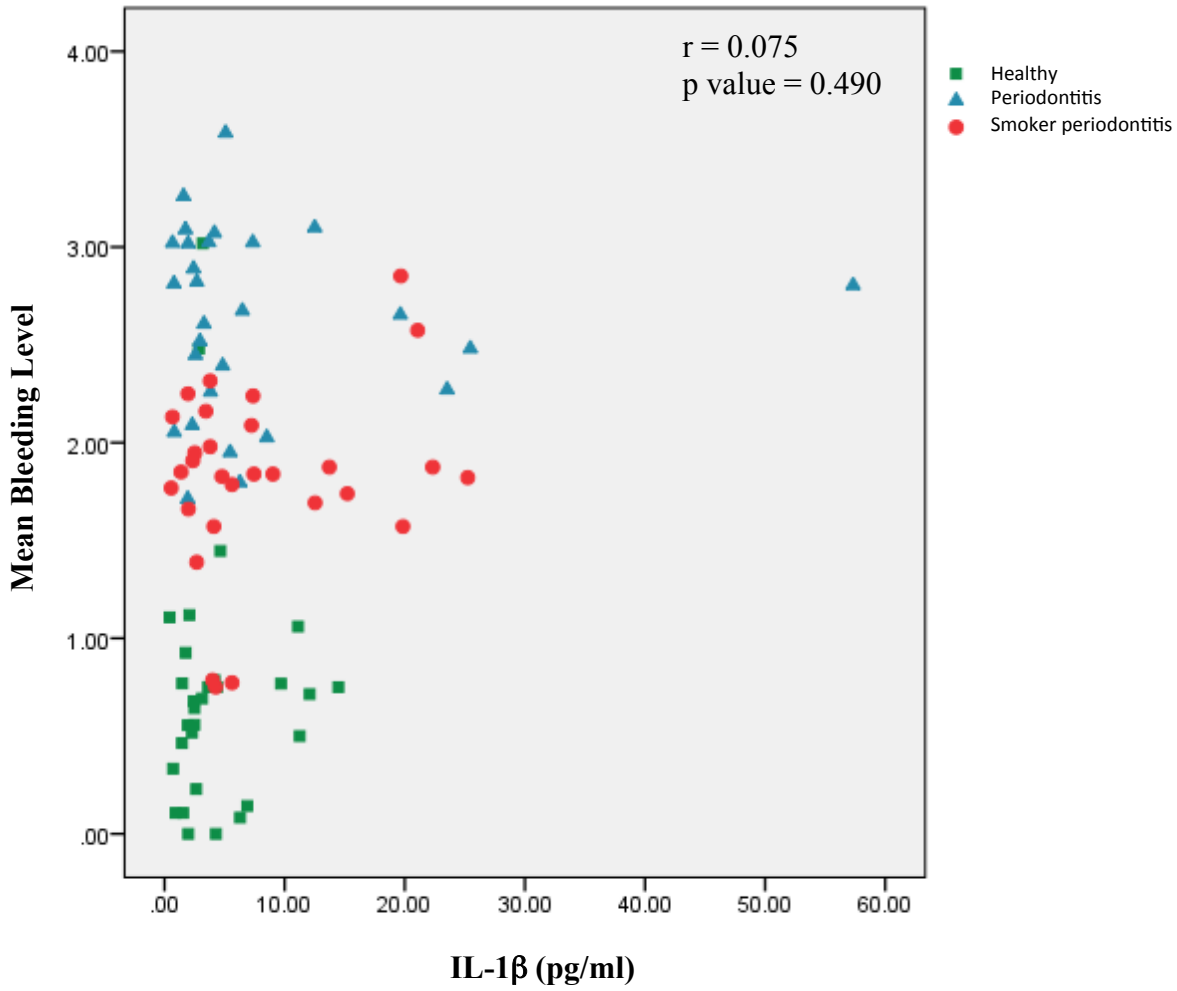


Figure7: Scatter plot of the bleeding and IL-1β level in saliva.

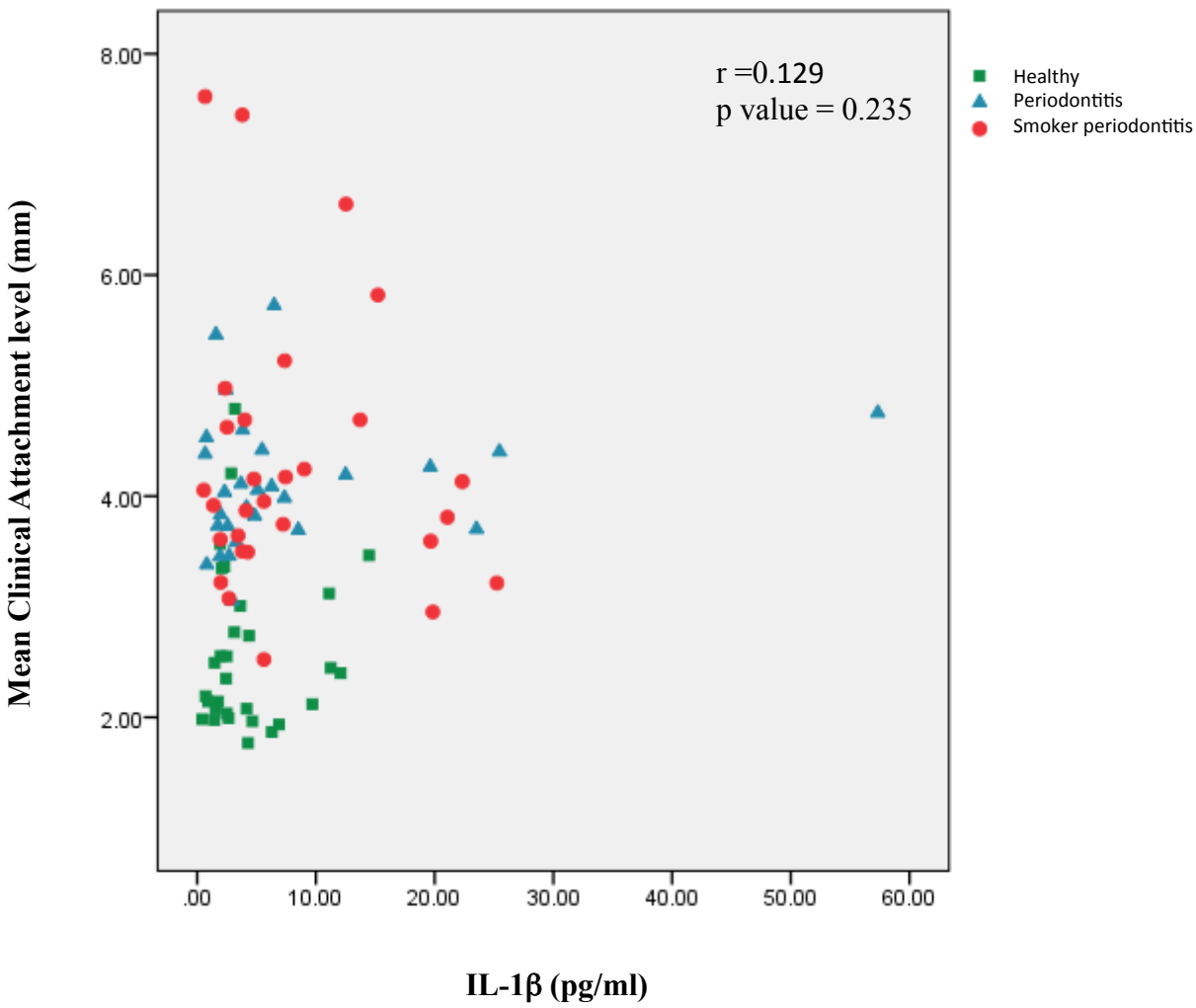


Figure 8: Scatter plot of the Clinical attachment level and IL-1β concentration in saliva.

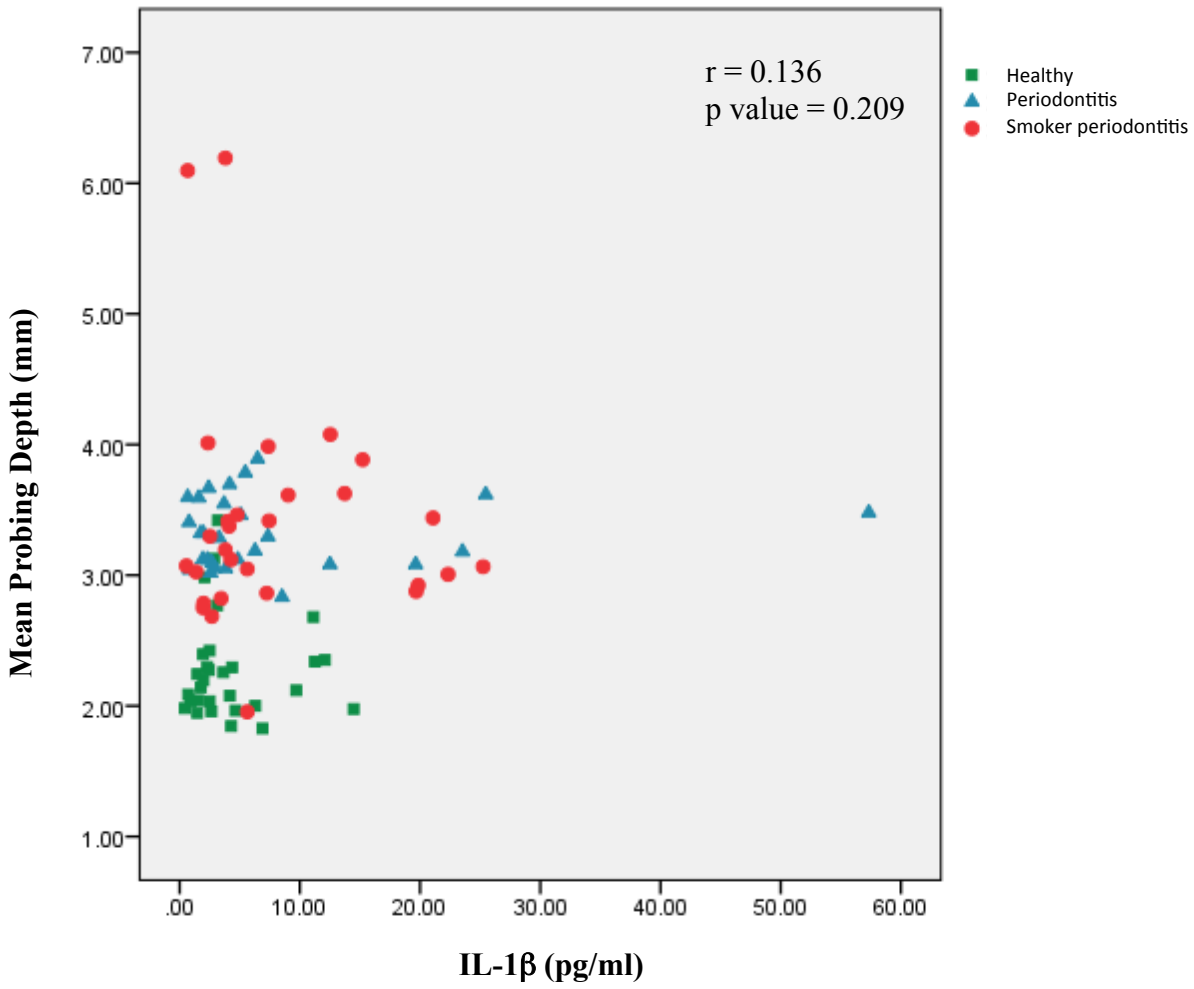


Figure 9: Scatter plot of the median probing depth and IL-1β concentration in saliva.

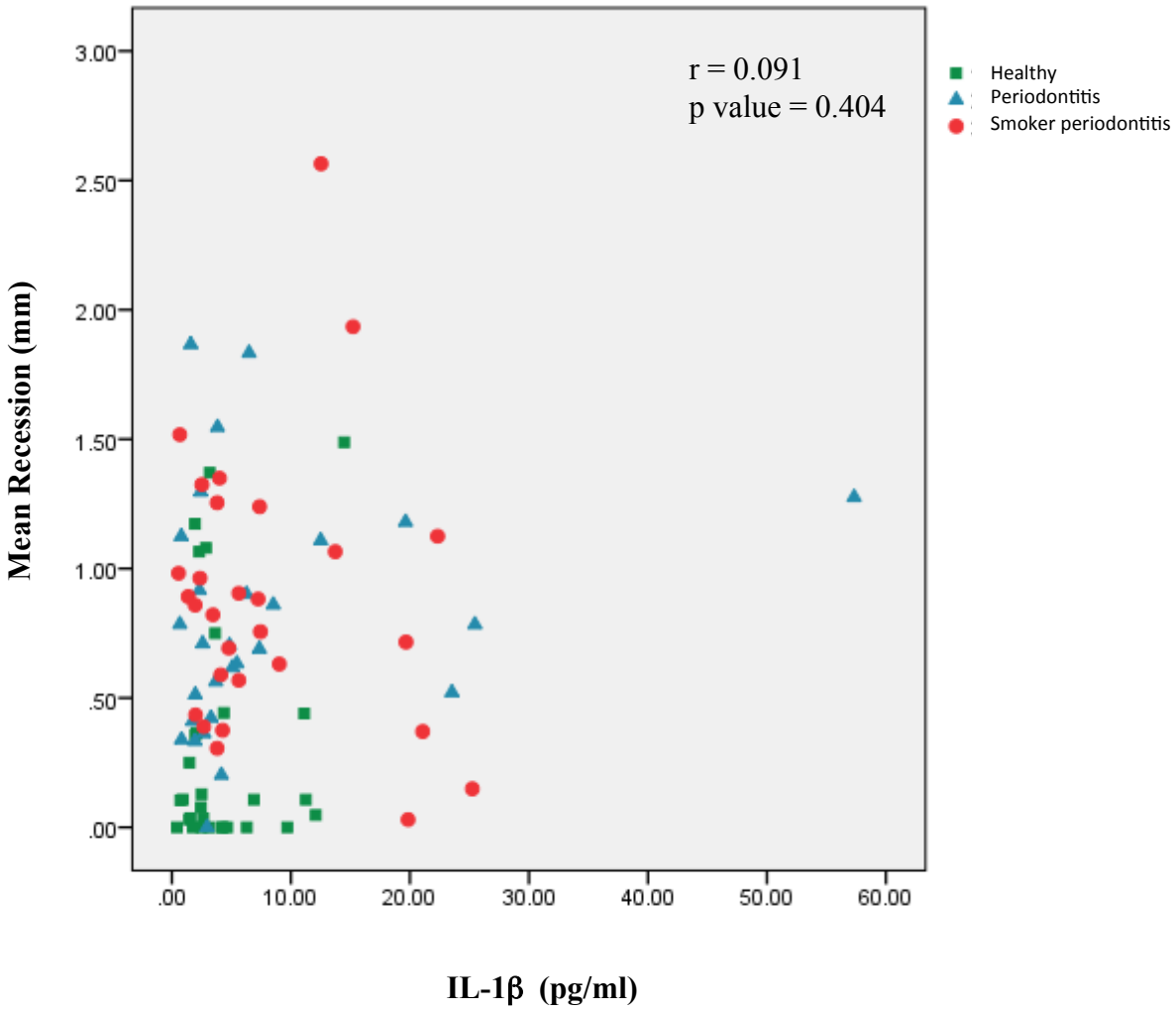


Figure 10: Scatter plot of the median recession and IL-1β concentration in saliva.

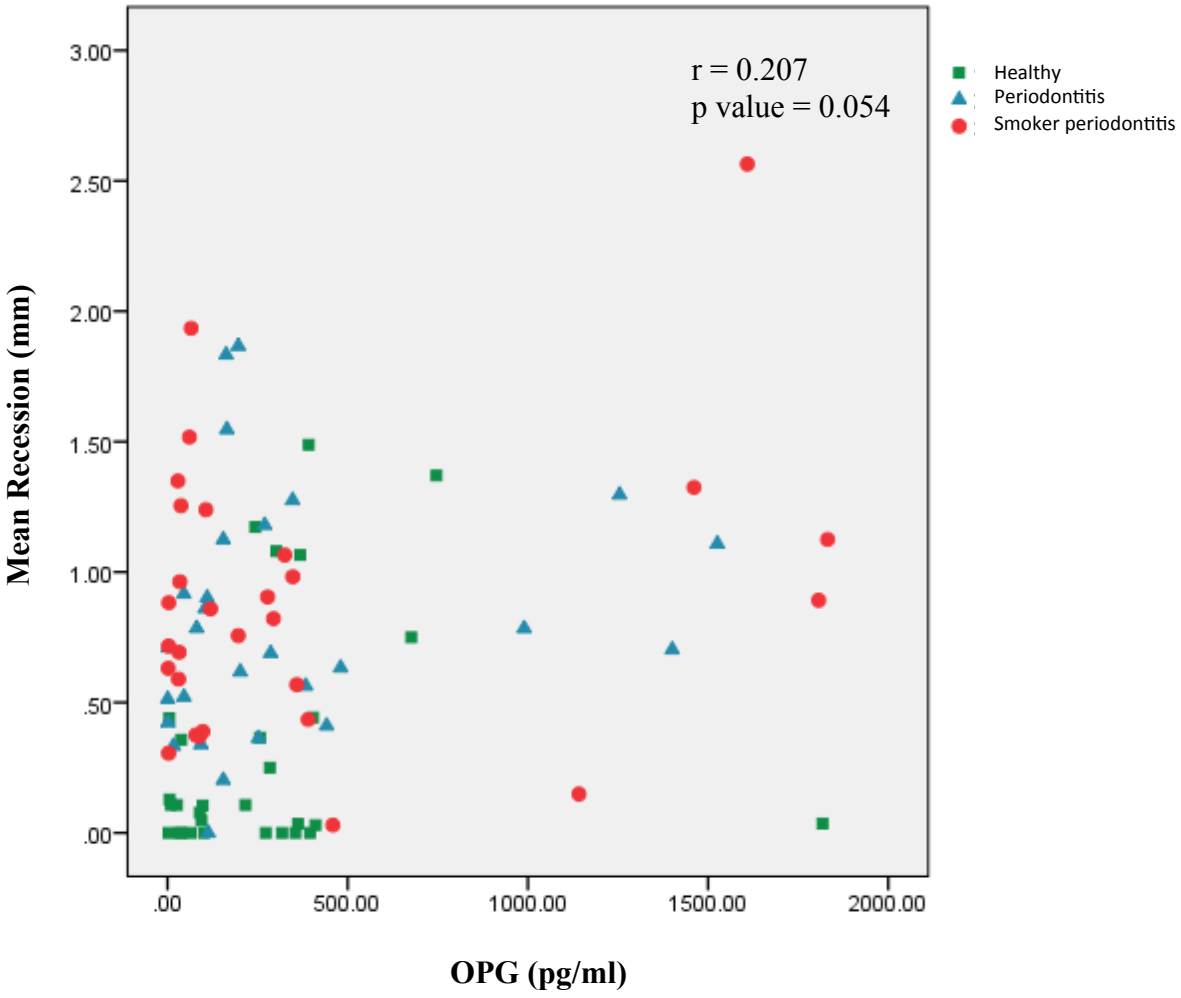


Figure 11: Scatter plot of the median recession and OPG concentration in saliva.

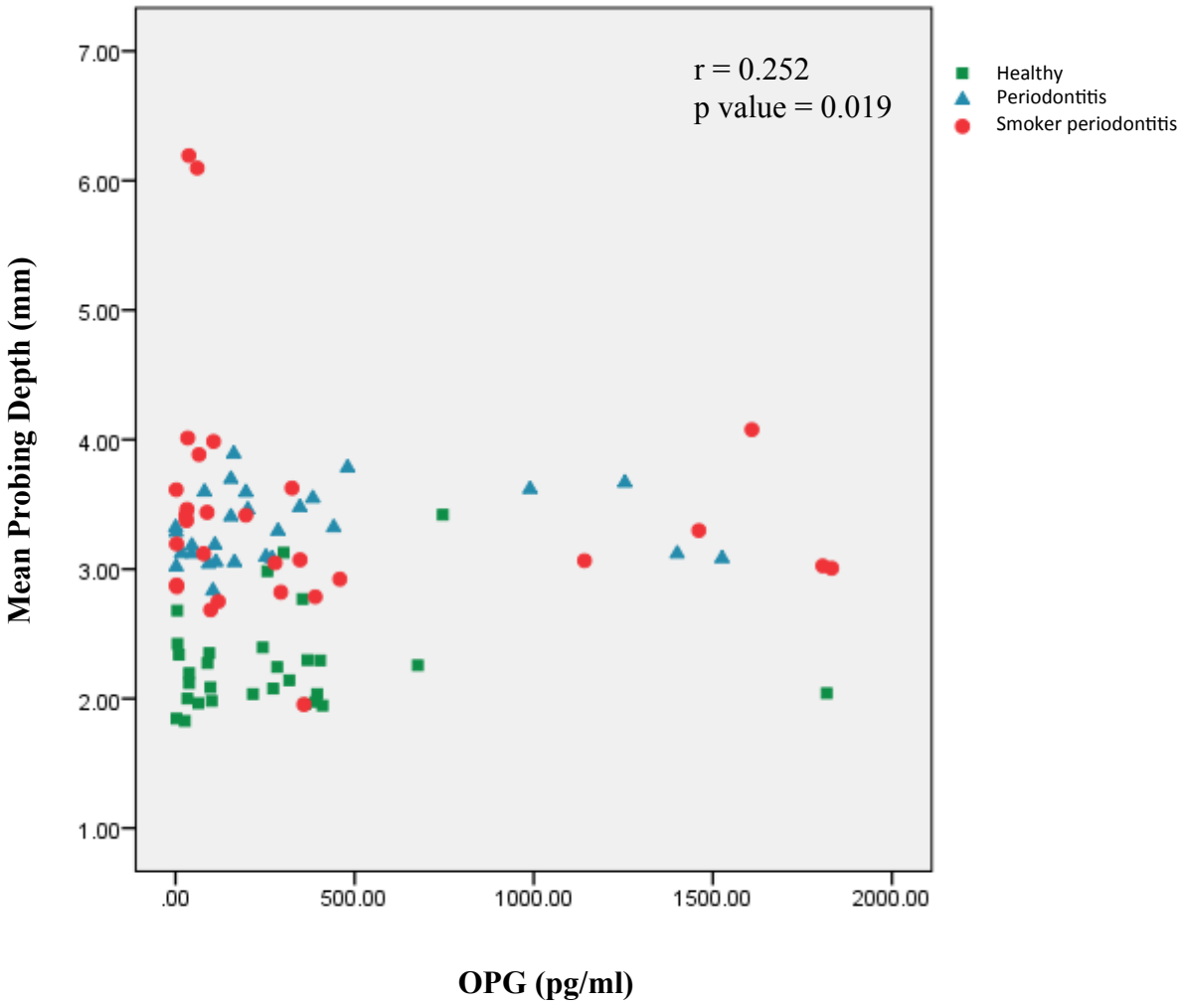


Figure 12: Scatter plot of the probing depth and OPG concentration in saliva.

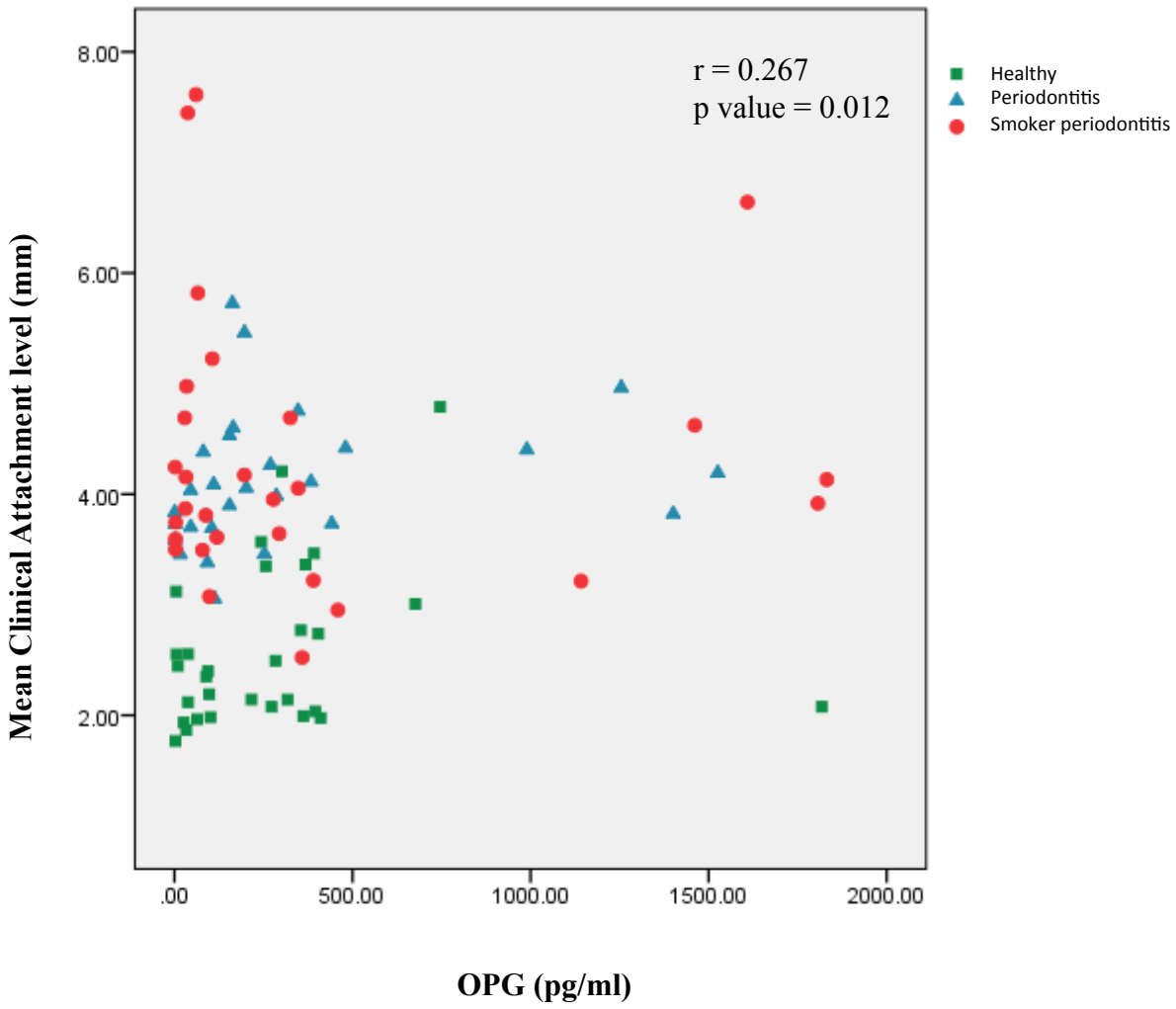


Figure 13: Scatter plot of the Clinical attachment level and OPG concentration in saliva.

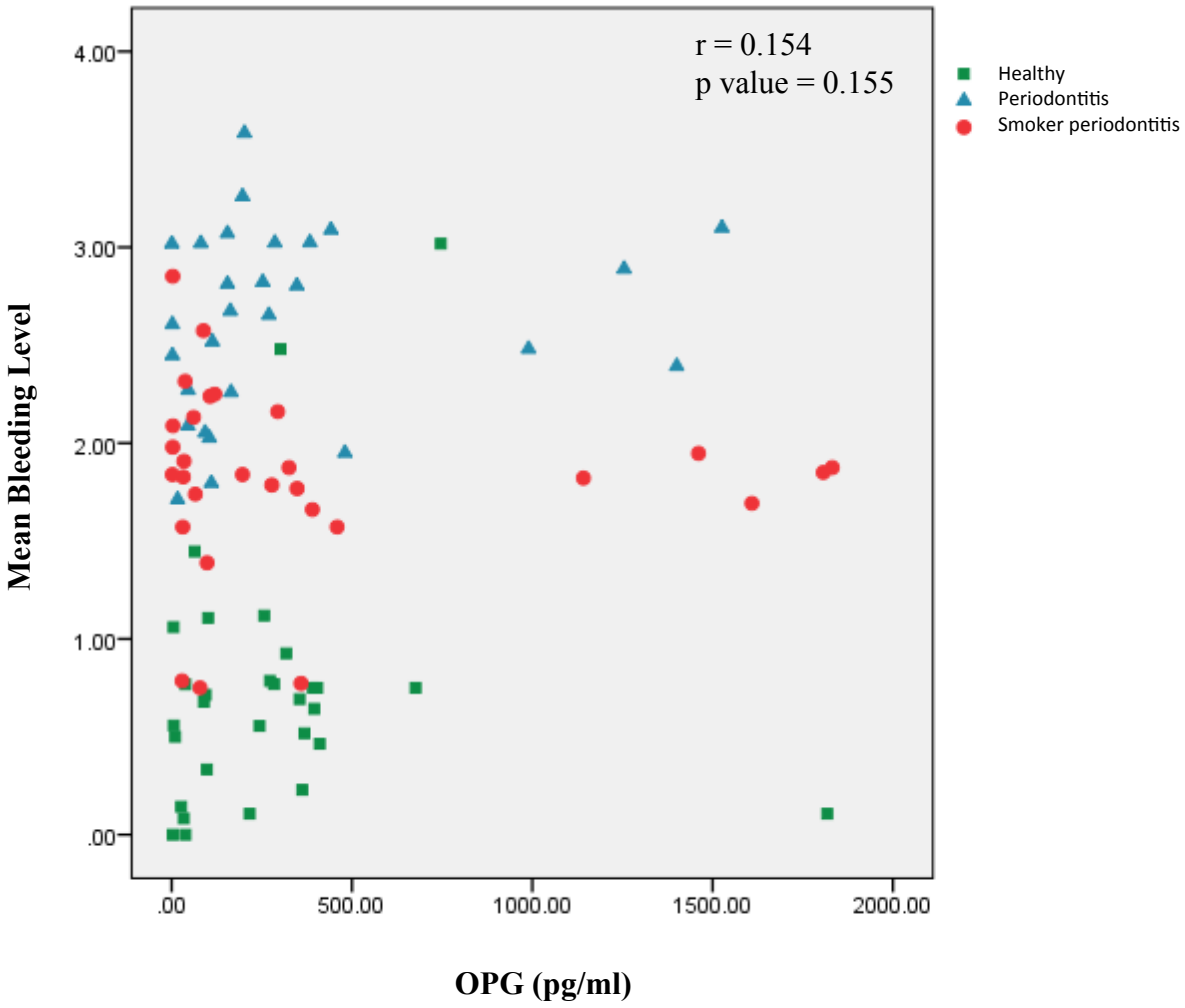


Figure14: Scatter plot of the Bleeding and OPG concentration in saliva.

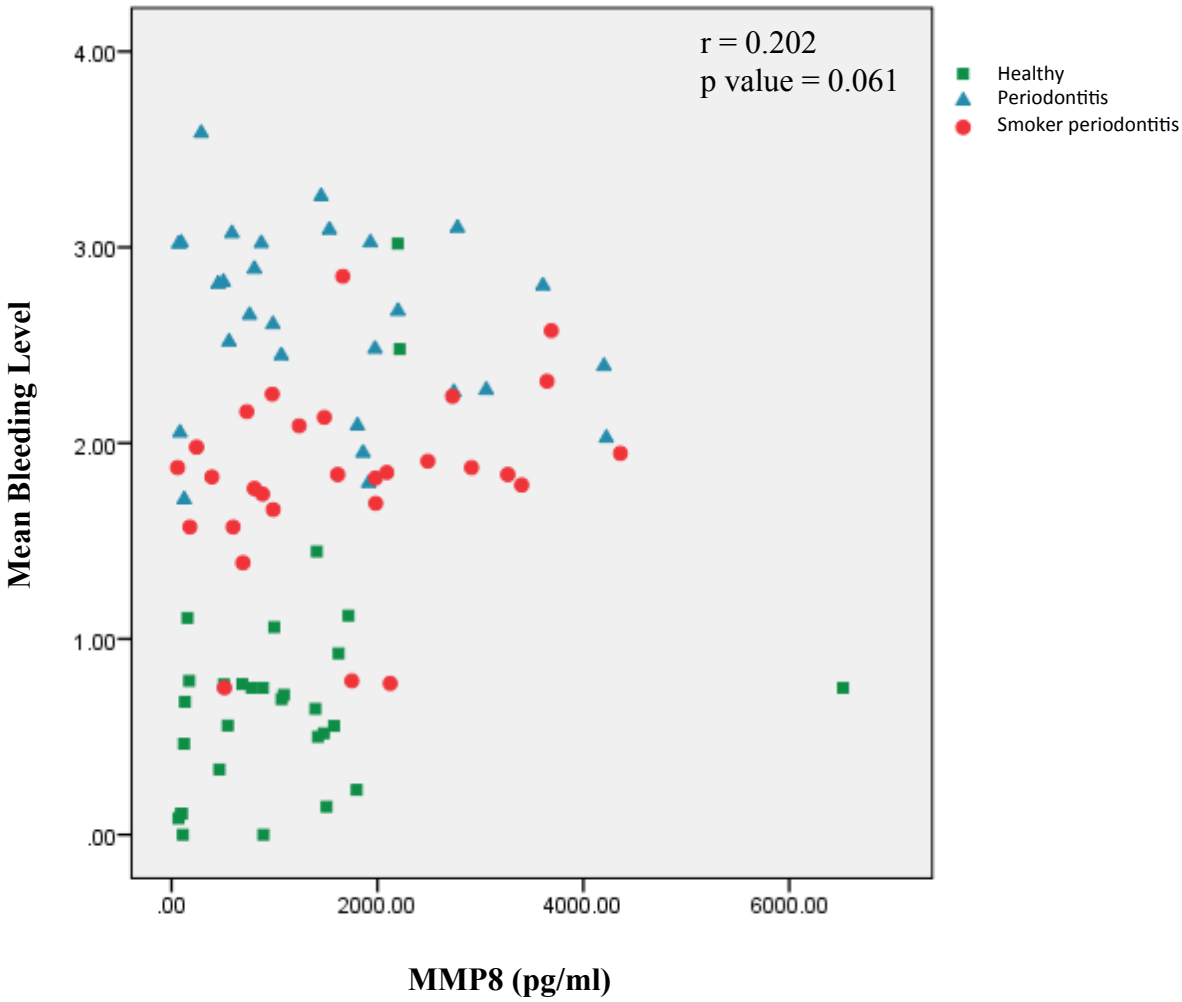


Figure15: Scatter plot of the Bleeding and MMP concentration in saliva.

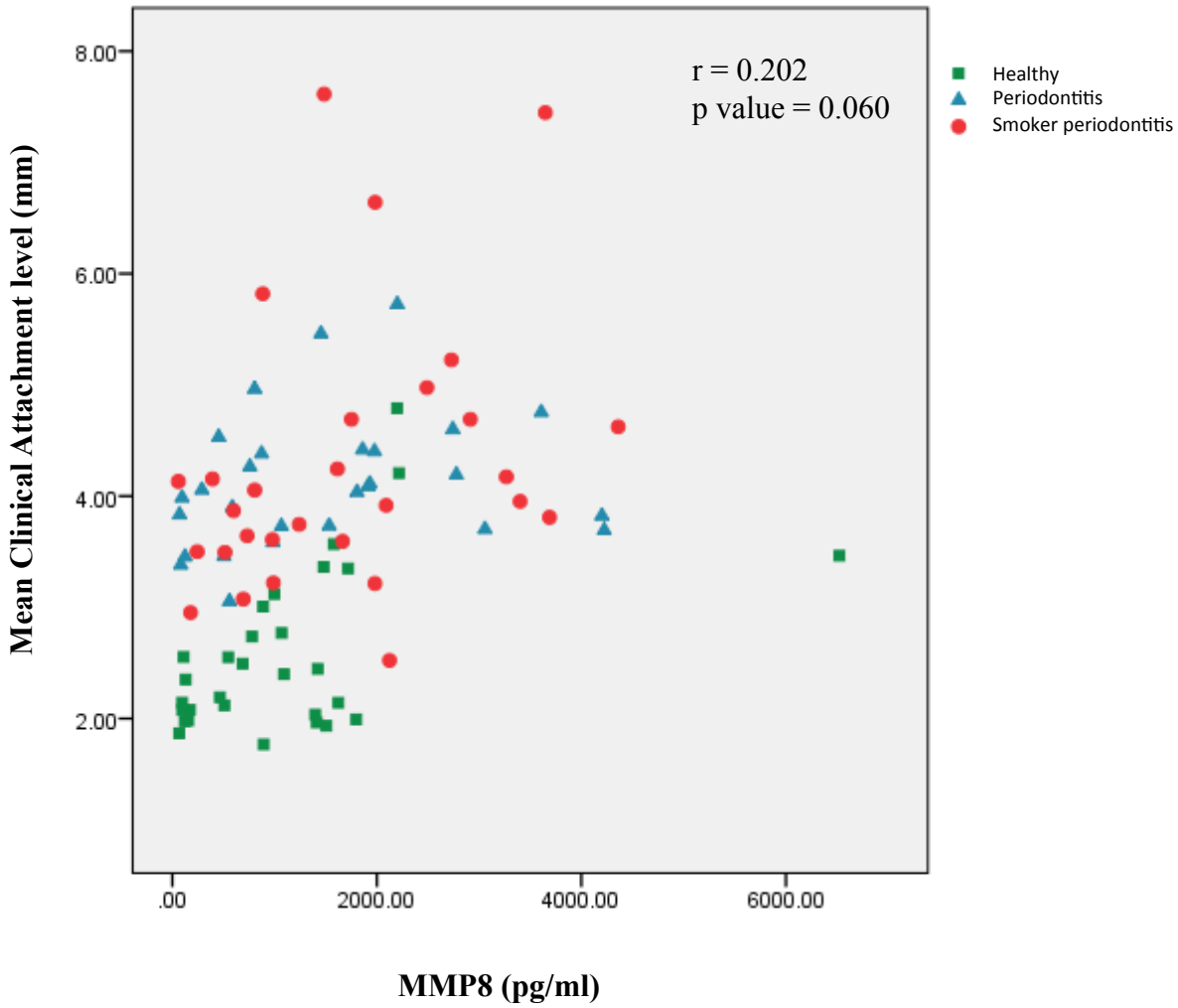


Figure 16: Scatter plot of the Clinical attachment level and MMP concentration in saliva.

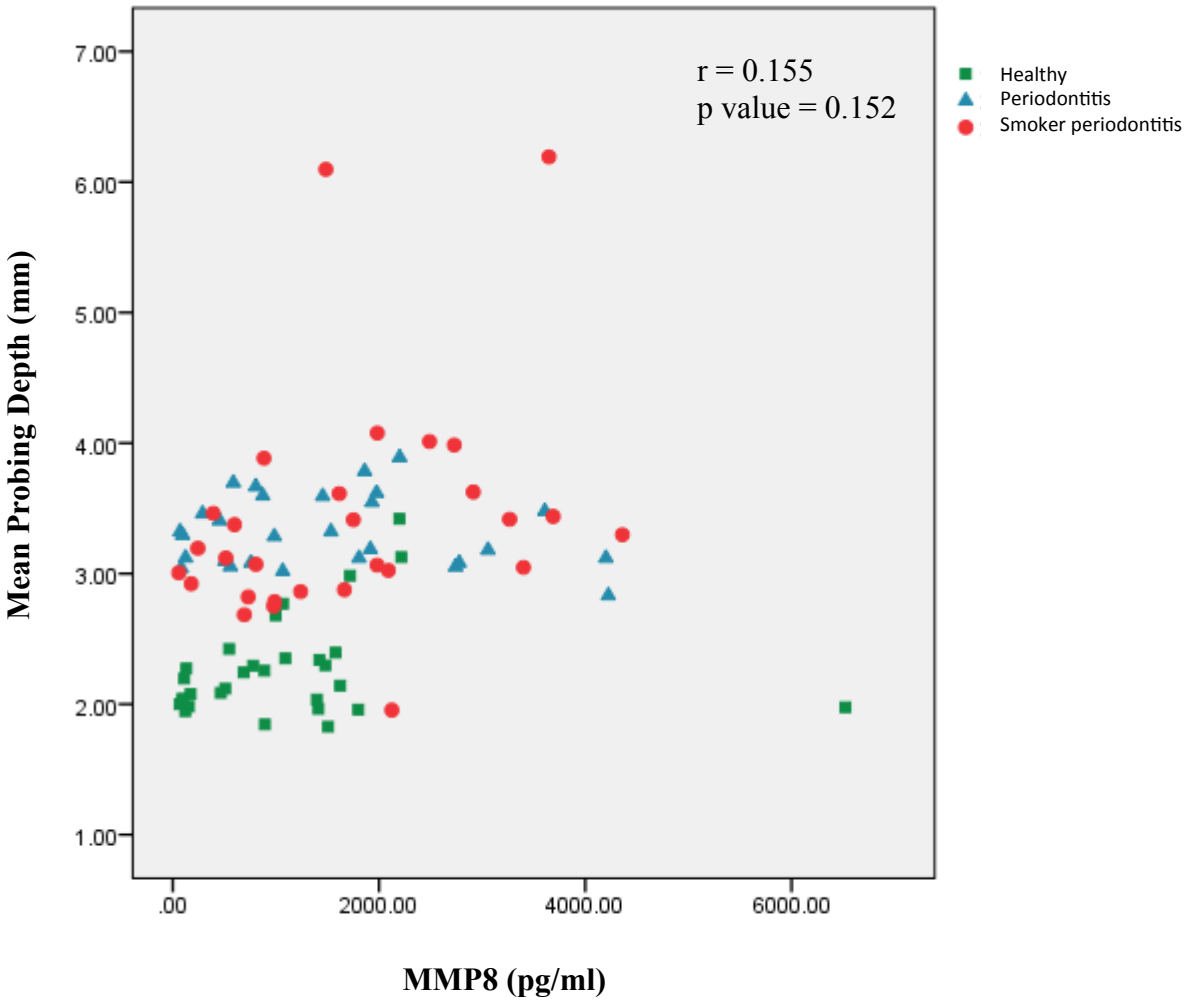


Figure 17: Scatter plot of the probing depth and MMP concentration in saliva.

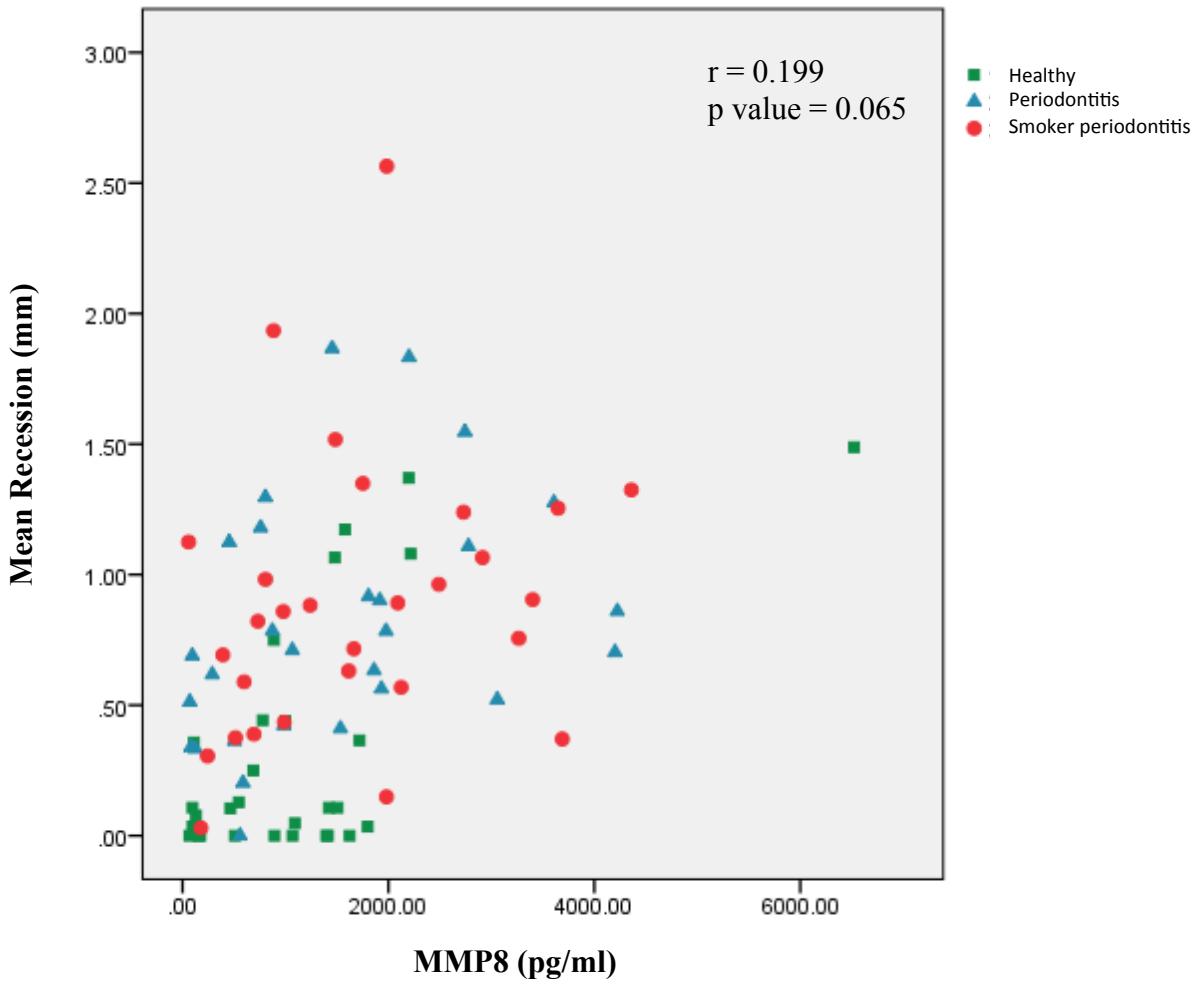


Figure 18: Scatter plot of the recession and MMP concentration in saliva .