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Article

Diagnostic Models for Screening of Chronic Periodontitis with Cytokines and Microbiologic Profiles in Saliva

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Abstract: This study was to investigate and assess salivary biomarkers as a means of diagnosing periodontitis. A total of 121 subjects were included: 28 periodontally healthy subjects, 24 with stage I, 24 with stage II, 23 with stage III, and 22 with stage IV periodontitis. Salivary proteins including active matrix metalloproteinase-8 (MMP-8), pro-MMP-8, total MMP-8, C-reactive protein, secretory immunoglobulin A and planktonic bacteria including Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola, Fusobacterium nucleatum, Prevotella intermedia, Porphyromonas nigrescens, Parvimonas micra, Campylobacter rectus, Eubacterium nodatum, Eikenella corrodens, Streptococcus mitis, Streptococcus mutans, Staphylococcus aureus, Enterococcus faecalis, and Actinomyces viscosus were measured from salivary samples. The performance of the diagnostic models was assessed by receiver operating characteristics (ROC) and area under the ROC curve (AUC) analysis. The diagnostic models were constructed based on the subjects' proteins and/or microbial profiles, resulting in two potential diagnosis models, which achieved better diagnostic powers with an AUC value > 0.750 for the diagnosis of stage II, III, and IV periodontitis (Model PC-I; AUC: 0.796, sensitivity: 0.754, specificity: 0.712) and for the diagnosis of stage III and IV periodontitis (Model PC-II; AUC: 0.796, sensitivity: 0.756, specificity: 0.868). This study can contribute to screening for periodontitis based on salivary biomarkers.

Keywords: periodontitis; diagnosis; saliva; biomarkers; matrix metalloproteinase; cytokines.

1. Introduction

The diagnosis of periodontitis is conventionally based on clinical evaluation, radiographic examinations, and periodontal parameters, including probing pocket depth, bleeding on probing, clinical attachment level, periodontal index and gingival index. However, conventional methods reflect the past evidence of inflammatory and infectious progression and the current extent and

severity of the chronic pathology. However, they do not address current activity or its episodic progression [1]. To overcome the aforementioned limitations, various tools for the detection of periodontitis have been developed and introduced [2,3].

The use of oral saliva as oral-based diagnostics has proven to be easy to use for point of care (POC) application. Oral-based diagnostics have been developed to detect several pathologies including oral cancer, human immunodeficiency virus infection, hepatitis C infection, and Ebola virus infection, with the advantages of being readily accessible and minimally invasive [4-7]. The ease of access and sampling of saliva containing inflammatory cytokines, microbial or viral infection provides potential possibilities for its use in the diagnosis of periodontitis [8].

It has been observed that there is an equilibrium in the interaction between the host and microorganisms when the periodontal apparatus is healthy [9]. This ecosystem can be affected by genetic factors of the host and environmental factors including smoking or diet [10]. The development of periodontal pathology is the outcome of several changes in the host or environmental state, indicating a possible relationship between the risk of periodontitis and susceptibility factors [11].

A previous study has demonstrated that microbiological variables could show a discriminating potential in distinguishing subjects with periodontitis [12]. Periodontal pathogens can induce the immune reaction of the host, resulting in the release of cytokines including matrix metalloproteinase-8 (MMP-8), C-reactive protein (CRP), secretory IgA (sIgA) [13-15]. A variety of these microbial profiles and inflammatory cytokines in saliva have been investigated as diagnostic tools for the detection of periodontitis. Although this diagnostic method has shown potential for identifying periodontitis, some limitations have been reported for this diagnostic tool that uses a single biomarker [8]. There was a study that showed an improvement of sensitivity and specificity in detection using a model that combined related biomarkers [16]. As periodontitis is a multifactorial disease, a model combining microbial profiles and cytokines involved in chronic infection might improve its diagnostic accuracy.

Recently, for the purpose of reflecting the severity, extent, and complexity of periodontal breakdown, a new classification of periodontitis has been suggested [17]: stage I, initial periodontitis; stage II, moderate periodontitis; stage III, severe periodontitis with potential for additional tooth loss; stage IV, advanced periodontitis with extensive tooth loss and potential for loss of dentition. However, little evidence has been accumulated in support of using salivary biomarkers for such a classification system.

The aim of this study was to verify alterations in salivary biomarkers, including microbial profiles and cytokines according to periodontal status, and to investigate a combined model for periodontitis in accordance with the new classification.

2. Materials and Methods

2.1. Subjects

This clinical study was approved by the Institutional Review Board of Seoul National University Dental Hospital. The subjects in this study were recruited from July 2019 to December 2019. Upon receiving written consent, 121 human subjects aged 18 years or older were evaluated at the Department of Periodontology in the Seoul National University Dental Hospital. All subjects involved in this study were required to have 20 or more teeth. In addition, the exclusion criteria applied was as follows: (a) use of any antibiotics or anti-inflammatory drugs within 3 months of registration; (b) use of an immunosuppressant (methotrexate, leflunomide, tacrolimus, cyclosporin, azathioprine) or adrenal cortical hormone (oral or injection) within 3 months of registration; (c) having less than 20 teeth; (d) having uncontrolled hypertension or diabetes; (e) subjects who have serious cardiovascular disease, respiratory system disease, kidney disease, liver disease, digestive system disease, blood system disease and neuropsychiatric disease; (f) subjects with hyperthyroidism or hypothyroidism; (g) women who were pregnant or planning to become pregnant; (h) subjects with autoimmune diseases; (i) subjects with a history or presence of malignant tumors in the jawbone; (j)

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subjects who have had a history of or are currently using drugs or alcohol abuse within one year; (k) subjects with other inflammatory diseases in the oral cavity besides periodontitis, such as stomatitis (including ulcerative, blistering, erosive), oral cancer; (l) subjects with other inflammatory diseases in the oral cavity besides periodontitis, such as ulcers, simple herpes and shingles, fungal or bacterial infections; and (m) subjects whose participation was judged by the researcher to be inappropriate because their involvement may cause ethical problems or seriously affect the research results.

2.2. Clinical and radiographic examination

A consent form was signed by and obtained from each subject following the sufficient explanation of the study. To identify if the subject was suitable for this study, demographic information such as gender and date of birth was obtained, and the systemic conditions of the participants were also examined.

On the first visit, clinical examinations were performed, and the following parameters were recorded. The gingival index (GI) [18]and plaque index (PI) [19]were examined on the buccal and lingual surfaces of the teeth. Additionally, the probing pocket depth (PPD), gingival recession (GR) and clinical attachment level (CAL) were measured at the six sites around the tooth. The amount of tooth loss (TL), sites of bleeding on probing (BOP), tooth mobility (TM) and furcation involvement (FI) were recorded per tooth. Alveolar bone loss at the mesial and distal site of the tooth was measured with periapical radiographs. The subjects were classified into healthy, and stages I, Ⅱ, Ⅲ, and Ⅳ depending on the severity, extent, and complexity of their periodontitis [17].

2.3. Preparation of solution for saliva storage

Approximately 0.1 M phenylmethylsulphonyl fluoride (PMSF) stock solution dissolved in isopropanol was stored at room temperature, and 0.5 M ethylenediaminetetraacetic acid (EDTA) stock solution dissolved in distilled water (DW) was stored refrigerated. The two stock solutions were stored independently, due to the instability of PMSF when mixed with 1X phosphate-buffered saline (PBS).

2.4. Whole saliva with draining method

Unstimulated saliva was collected with the participant's head tilting slightly forward in a sitting position by drooling into a funnel-shaped test tube. The sampling was performed for 15 minutes and was stopped when the amount collected reached 5 ml. Subsequently, the saliva sample was placed on ice, and supplemented with 1X PBS 4930μl, 20 μl EDTA solution, and 50 μl PMSF stock solution; then, vortexing was performed. The samples were stored in a deep freezer at a temperature of -80 ℃ immediately after collection for the preservation of biomarkers.

2.5. Saliva collection for oral microbial identification

After collecting GCF, subjects took another break for 5 minutes. The subjects gargled and rinsed with gargle solution (EasyperiO kit, YD Life Science Company, Gyeonggi-do, Korea) for 30 seconds, and then spit it into the sample container. The sample container cap was closed tightly. Samples were stored in a refrigerator (4 ℃) and transported to the analytical company (YD Life Science company, Gyeonggi-do, Korea) under dry ice.

2.6. Protein Biomarker Assays

Protein biomarker levels were detected with colorimetric-based enzyme-linked immunosorbent assays (ELISAs) for measurement of active MMP-8, pro-MMP-8, total MMP-8, CRP, and sIgA according to the manufacturer's protocols (Sugentech, Osong, Korea). Prior to performing the assay, 1 ml of whole saliva was passed through a 0.45-μm syringe filter to obtain cell-free supernatant. After diluting the sample at a predetermined ratio, 75 μl of the diluted solution was dispensed into the sample inlet using a micropipette. After 10 minutes, absorbance measurements were collected using a fluorescence measurement device (Sugentech, Osong, Korea).

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2.7. Detection of microbiologic profiles

The samples were analyzed by quantitative analysis using multiplex-quantity real time polymerase chain reaction (PCR) according to the manufacture's protocol (EasyPerio; YD life science, Gyeonggi-do, Korea) for 16 oral pathogenic bacteria: Aggregatibacter actinomycetemcomitans(A. actinomycetemcomitans, Aa), Porphyromonas gingivalis(P. gingivalis, Pg), Tannerella forsythia (T. forsythia, Tf), Treponema denticola (T. denticola, Td), Fusobacterium nucleatum (F. nucleatum, Fn), Prevotella intermedia (P. intermedia, Pi), Porphyromonas nigrescens (P. nigrescens, Pn), Parvimonas micra (P. micra, Pm), Campylobacter rectus (C. rectus, Cr), Eubacterium nodatum (E. nodatum, En), Eikenella corrodens (E. corrodens, Ec), Streptococcus mitis (S. mitis, Sm), Streptococcus mutans (S. mutans, Smu), Staphylococcus aureus (S. aureus, Sa), Enterococcus faecalis (E. Faecalis, Ef), and Actinomyces viscosus (A. viscosus, Av). Patient-based microbiologic data was analyzed.

2.8. Statistical Analysis

Continuous data was represented with means and standard deviation for each subject group. Group comparisons were made using one-way ANOVA in SPSS version 17 (IBM Software, Armonk, New York, USA). Dichotomized data was represented with number and percentage for each subject group. Group comparisons were made with a Fisher's exact test. Differences were considered statistically significant when the P-value < 0.05. Seven diagnostic models of periodontitis (Model PD), stage I periodontitis (Model PD-I), stage II periodontitis (Model PD-II), stage III periodontitis (Model PD-III), stage IV periodontitis (Model PD-IV), periodontitis above stage I (stage II, III, and IV; Model PA-I), and periodontitis above stage II (stage III and IV; Model PA-II) were constructed according to the concentration of proteins and microbial profiles based on a forward stepwise logistic regression analysis using SPSS statistics 21.0 software. The diagnostic models were evaluated by sensitivity, specificity, and ROC curve analysis using Excel (Microsoft 365, Redmond, Washington, USA).

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3. Results

3.1. Demographic and clinical characteristics of subjects

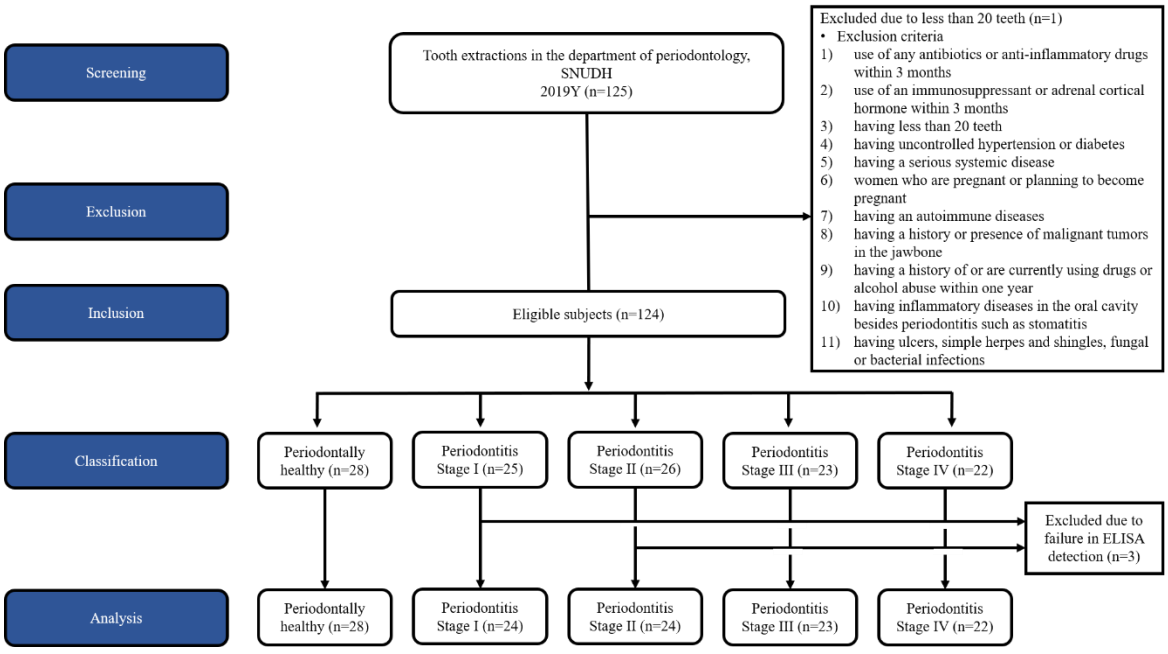

Figure 1. CONSORT flow diagram of the study.

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Thirty-eight male (30.4%) and eighty-seven female (69.6%) subjects, ranging in age from 20 to 79 years, were enrolled in the study. Following the recording of clinical and radiographic parameters, the subjects were allocated into the five groups of periodontal status. One subject was excluded due to lack of teeth. The data of three subjects were excluded because the proteins including active MMP-8, pro-MMP-8, total MMP-8 did not show detectable levels in the whole saliva sample. A total of 121 subjects were included in the final analysis (Figure 1). The analysis of the data obtained from the healthy (n = 28), and periodontitis population (n = 93; stage I: 24 stage II: 24 stage III: 23 and stage IV: 22) were performed in this study.

Table 1. Demographic characteristics, clinical periodontal parameters, salivary biomarkers and planktonic bacteria of each group (mean ± standard deviation).

	Healthy Controls (HC) (n=28)	Periodontitis (PD)				Significance
		PS-I (n=24)	PS-II (n=24)	PS-III (n=23)	PS-IV (n=22)	
Age	30.04 ± 8.79	35.00 ± 15.10	49.21 ± 16.92	58.17 ± 14.40	61.41 ± 11.35	< 0.001
Sex						
Male	4 (14.3%)	3 (12.5%)	11 (45.8%)	9 (39.1%)	10 (45.5%)	0.010
Female	24 (85.7%)	21 (87.5%)	13 (54.2%)	14 (60.9%)	12 (54.5%)	
Hypertension	0	1 (4.2%)	1 (4.2%)	4 (17.4%)	6 (27.3%)	0.005
Diabetes	0 (0.0%)	1 (4.2%)	0 (0.0%)	2 (8.7%)	4 (18.2%)	0.023
Osteoporosis	0 (0.0%)	1 (4.2%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0.769
Hepatitis	0 (0.0%)	0 (0.0%)	1 (4.2%)	0 (0.0%)	0 (0.0%)	0.769
Smoking	0 (0.0%)	0 (0.0%)	2 (8.3%)	4 (17.4%)	1 (4.5%)	0.032
# of teeth	27.68 ± 1.467	27.44 ± 1.227	27.46 ± 1.560	27.56 ± 1.635	26.59 ± 1.681	0.12
BOP, %	30 ± 18	37 ± 19	28 ± 20	35 ± 23	32 ± 24	0.595
Mean GI	0.58 ± 0.57	0.66 ± 0.45	0.50 ± 0.39	0.66 ± 0.69	0.75 ± 0.55	0.592
Mean PI	0.48 ± 0.41	0.51 ± 0.45	0.42 ± 0.45	0.73 ± 0.58	0.61 ± 0.51	0.184
Mean PPD	2.36 ± 0.26	2.54 ± 0.30	2.54 ± 0.33	2.70 ± 0.56	2.83 ± 0.62	0.003
Mean GR	0.11 ± 0.33	0.053 ± 0.056	0.12 ± 0.15	0.26 ± 0.27	0.65 ± 0.97	< 0.001
Mean CAL	0.24 ± 0.58	0.12 ± 0.12	0.27 ± 0.29	0.57 ± 0.59	1.25 ± 1.43	< 0.001
Mean ABL	0.077 ± 0.32	0.22 ± 0.16	0.50 ± 0.27	1.18 ± 0.71	2.34 ± 2.07	< 0.001
# of FI	0	0	0	0.36 ± 0.78	0.73 ± 1.32	< 0.001
TM0	27.25 ± 2.45	27.20 ± 1.35	27.29 ± 1.33	26.36 ± 1.98	24.09 ± 3.31	< 0.001
TM1	0	0	0	0.80 ± 1.38	1.64 ± 2.08	< 0.001
TM2	0	0	0	0	0.27 ± 0.63	0.001
TM3	0	0	0	0.04 ± 0.20	0.23 ± 0.61	0.022
Sal. Ez. (ng/mL)						
Active MMP-8	119.10 ± 93.73	172.82 ± 166.54	190.57 ± 125.68	281.87 ± 206.93	288.35 ± 145.54	< 0.001
Pro-MMP -8	115.90 ± 90.62	127.38 ± 82.44	155.02 ± 93.70	217.72 ± 104.60	256.90 ± 139.43	< 0.001
Total MMP-8	235.01 ± 178.71	300.21 ± 233.94	345.59 ± 209.98	499.57 ± 286.48	545.24 ± 270.87	< 0.001
CRP	0.93 ± 3.01	0.82 ± 1.68	0.47 ± 1.18	0.70 ± 1.10	0.37 ± 0.80	0.564
sIgA	225.16 ± 135.68	221.45 ± 133.02	221.25 ± 115.93	321.20 ± 163.72	336.21 ± 184.33	0.012

Abbreviations: PS-I: stage-I periodontitis; PS-II: stage-II periodontitis; PS-III: stage-III periodontitis; PS-IV: stage-IV periodontitis; BOP: bleeding on probing; GI: gingival inflammation; PI: plaque index; PPD: probing pocket depth; GR: gingival recession; CAL: clinical attachment loss; ABL: alveolar bone loss; FI: furcation-

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involved tooth; TM0: immobile tooth°; TM1: mobile tooth with 1°; TM2: mobile tooth with 2°; TM3: mobile tooth with 3°; Sal. Ez.: salivary enzyme; MMP: matrix metalloproteinase; CRP: C-reactive protein; sIgA: secretory IgA.

The demographic data (Table 1) for systemic disease, including osteoporosis and hepatitis, were balanced among the five groups. However, age, sex, hypertension, diabetes mellitus and smoking were statistically and significantly different among the groups. The subjects with hypertension, diabetes and smoking were not found in the periodontally healthy group; however, they were significantly associated with stage III and IV periodontitis. In addition, the older participants were significantly found the higher stage of periodontitis.

Dental and periodontal data (Table 1) were significantly different among the five groups for mean GR (0.11 to 0.65; $P < 0.001$), mean PPD (2.36 to 2.83 mm; $P = 0.003$), mean CAL (0.24 to 1.25 mm; $P < 0.001$), mean MBL (0.077 to 2.34 mm; $P < 0.001$), number of furcation-involved teeth (0 to 0.73; $P<0.001$), number of immobile teeth (24.09 to 27.25; $P < 0.001$), number of mobile teeth with grade 1 (0 to 1.64; $P < 0.001$), number of mobile teeth with grade 2 (0 to 0.27; $P = 0.001$), and number of mobile teeth with grade 3 (0 to 0.23; $P = 0.022$).

3.2. Cytokines and microbial profiles in saliva

The data of proteins and microbiologic profiles are shown in Table 1. The levels of protein concentrations of active MMP-8 ($P < 0.001$), pro-MMP-9 ($P < 0.001$), and total MMP-8 ($P < 0.001$) and sIgA ($P = 0.012$) showed significant differences among the groups. The level of P. gingivalis ($P = 0.012$), T. forsythia ($P = 0.005$), T. denticola ($P = 0.028$), P. micra ($P < 0.001$), C. rectus ($P = 0.001$), E. nodatum ($P < 0.001$) and A. viscosus ($P = 0.023$) showed significant differences among the groups. The overall microbial profiles are presented in Figure 2.

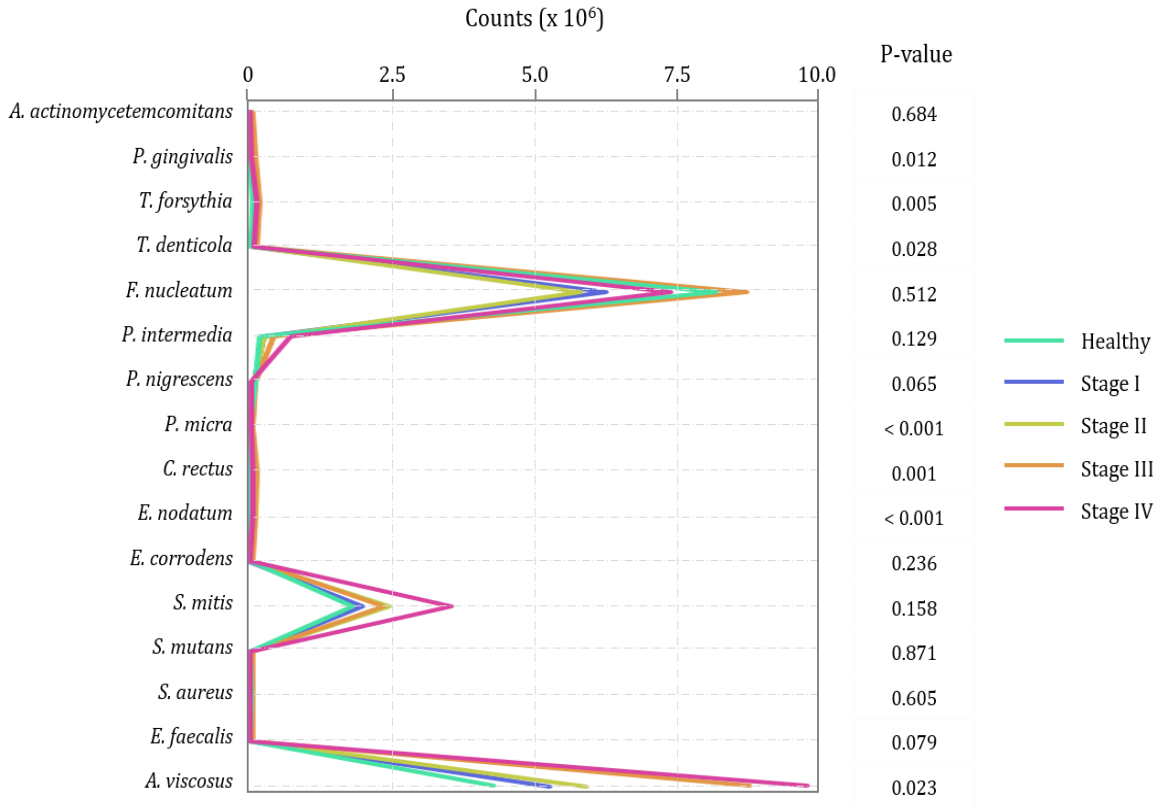

Figure 2. Changes in profiles of salivary bacteria according to stages of periodontitis (×10⁶)

3.3. Construction of Diagnostic Models Based on Cytokines in Saliva

Diagnostic models of periodontitis (Model PD), stage I periodontitis (Model PD-I), stage II periodontitis (Model PD-II), stage III periodontitis (Model PD-III), stage IV periodontitis (Model PD-

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IV), periodontitis above stage I (stage II, III, and IV; Model PA-I), and periodontitis above stage II (stage III, and IV; Model PA-II) were constructed using the proteins and microbial profiles that showed significant differences.

Table 2. The sensitivity, specificity, and accuracy of each prediction model.

Total	Clinical final diagnosis		Prediction Model		FP	FN	Sensitivity		Specificity		Accuracy	
121	HC	28	-		-	-	-		-		-	
	Periodontitis	93	PD	117	24	0	1.000	(93/93)	0.143	(4/28)	0.802	(97/121)
	PS-I	24	PD-I	4	3	23	0.042	(1/24)	0.969	(94/97)	0.785	(95/121)
	PS-II	24	PD-II	5	5	24	0.000	(0/24)	0.948	(92/97)	0.760	(92/121)
	PS-III	23	PD-III	11	4	16	0.304	(7/23)	0.959	(94/98)	0.835	(101/121)
	PS-IV	22	PD-IV	9	2	15	0.318	(7/22)	0.980	(97/99)	0.860	(104/121)
	PS-II, III, & IV	69	PA-I	67	15	17	0.754	(52/69)	0.712	(37/52)	0.736	(89/121)
	PS-III & IV	45	PA-II	44	10	11	0.756	(34/45)	0.868	(66/76)	0.826	(100/121)

Abbreviations: HC: healthy controls; PS-I: stage-I periodontitis; PS-II: stage-II periodontitis; PS-III: stage-III periodontitis; PS-IV: stage-IV periodontitis; FP: False positive; FN: False negative.

The Model PD mathematical formula with an accuracy of 0.802 (Table 2) was constructed to discriminate the periodontitis groups from the healthy group using a logistic regression analysis. This model showed high sensitivity (1.000), but low specificity (0.143) (Table 2).

$$\text{Model PD} = \frac{1}{1 + e^{-(-0.066 + 0.002*\text{T-MMP8} + 0.505*\text{En})}}$$

The Model PD-I mathematical formula with an accuracy of 0.785 (Table 2) was constructed to diagnose the stage I periodontitis group from the healthy group and stage II, III, and IV periodontitis patients using a logistic regression analysis. This model showed high specificity (0.969) but low sensitivity (0.042) (Table 2).

$$\text{Model PD-I} = \frac{1}{1 + e^{-(-0.825 - 10.113*\text{Active-MMP8} - 10.125*\text{Pro-MMP8} + 10.117*\text{Total-MMP8})}}$$

The Model PD-II mathematical formula with an accuracy of 0.760 (Table 2) was constructed to diagnose the stage II periodontitis group from the healthy group and stage I, III, and IV periodontitis patients using logistic regression analysis. This model showed high specificity (0.948) but low sensitivity (0.000) (Table 2).

$$\text{Model PD-II} = \frac{1}{1 + e^{-(-1.308 - 0.513*\text{Pm} + 0.293\ \text{Cr})}}$$

The Model PD-III mathematical formula with an accuracy of 0.835 (Table 2) was constructed to diagnose the stage III periodontitis group from the healthy group and stage I, II, and IV periodontitis patients using logistic regression analysis. This model showed high specificity (0.959) but low sensitivity (0.304) (Table 2).

$$\text{Model PD-III} = \frac{1}{1 + e^{-(-2.946 + 10.998*\text{Active-MMP8} + 10.999*\text{Pro-MMP8} - 10.999*\text{Total-MMP8} - 0.259*\text{Td} + 0.419*\text{Pm} + 0.419*\text{En})}}$$

The Model PD-IV mathematical formula with an accuracy of 0.860 (Table 2) was constructed to diagnose the stage IV periodontitis group from the healthy group and stage I, II, and III

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periodontitis patients using logistic regression analysis. This model showed high specificity (0.980) but low sensitivity (0.318) (Table 2).

$$\text{Model PD-IV} = \frac{1}{1 + e^{-(-12.023 + 0.004*\text{Pro-MMP8} + 0.427*\text{En} + 1.267*\text{Av})}}$$

The Model PA-I mathematical formula with an accuracy of 0.736 (Table 2) was constructed to discriminate the stage II, III, and IV periodontitis groups from the healthy group and stage I periodontitis patients using logistic regression analysis. This model showed improved sensitivity (0.754) and specificity (0.712) (Table 2).

$$\text{Model PA-I} = \frac{1}{1 + e^{-(-1.635 + 0.005*\text{Pro-MMP8} + 0.193*\text{Cr} + 0.322*\text{En})}}$$

The Model PA-II mathematical formula with an accuracy of 0.826 (Table 2) was constructed to discriminate the stage III and IV periodontitis groups from the healthy group and stage I and II periodontitis patients using logistic regression analysis. This model showed good specificity (0.756) and sensitivity (0.868) (Table 2).

$$\text{Model PA-II} = \frac{1}{1 + e^{-(-11.310 + 0.003*\text{sIgA} + 0.300*\text{Pg} - 0.302*\text{Tf} + 0.324*\text{Pm} + 0.675*\text{En} + 1.231*\text{Av})}}$$

3.4. Validation of multianalyte models

Among the seven models, Model PA-I and Model PA-II, which showed good sensitivity and specificity, were further investigated with the ROC curves (Figure 3).

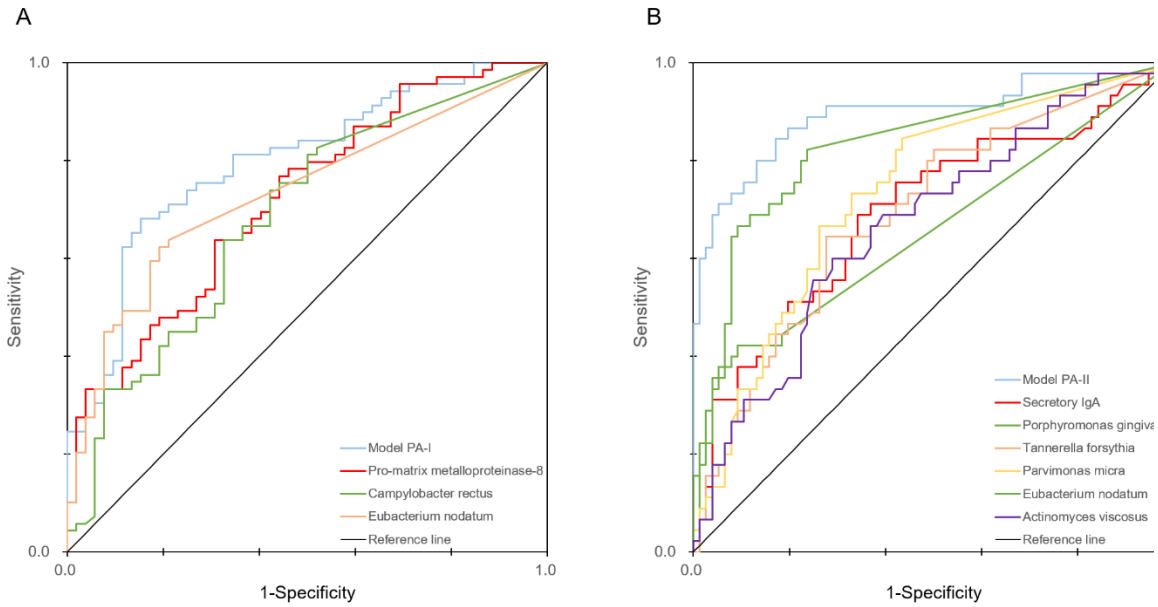

Figure 3. ROC curve of models above PA-I (A) and II (B). ROC curve of biomarkers significantly related and included in each model were also included in each figure.

It was found that the models combining salivary biomarkers and microbiologic profiles were useful for discriminating periodontal status. The AUC values of pro-MMP-8, C. rectus, and E. nodatum were 0.720, 0.685, and 0.733, respectively. In contrast, the AUC value of model PA-I that combined the markers showed that the 0.796. AUC values of sIgA, P. gingivalis, T. forsythia, P. micra, E. nodatum, and A. viscosus were 0.695, 0.653, 0.694, 0.736, 0.831, and 0.673, respectively, while

Model PA-II that combined them showed an AUC value of 0.894.This section may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation as well as the experimental conclusions that can be drawn.

4. Discussion

In this study, we built the seven diagnostic models for periodontitis by combining meaningful salivary biomarkers including cytokines and microbial profiles which significantly changed with the stage of periodontitis. Among the seven models, PA-I and PA-II were both highly sensitive and specific compared with PD-I, II, III and IV models. In model PA-I, E. nodatum and C. rectus were included; these bacteria belong to the orange complex, which has a role in linking early colonizing and pathogenic bacteria of chronic periodontitis. Therefore, it is thought that these bridging species are very important in the diagnosis of periodontitis with stage II, III, and IV disease. Meanwhile, in model PA-II, purple complex such as A. viscosus, orange complex such as P. micra and E. nodatum, red complex such as P. gingivalis and T. forsythia were included. The high accuracy of model PA-II means that overall planktonic bacterial species can increase when the severity of periodontitis increases, because various species consisting of early colonizer, bridging species, and pathogenic bacteria are included. Simple counting of bacteria also showed that there were more group of streptococcus and A. viscosus in stage IV, meaning the more severe the stage of periodontitis is, the more overall bacteria there are.

To our knowledge, there is little literature to validate a new classification system of periodontitis in the development of a diagnostic method including salivary biomarkers. In 2017, the new classification system for case definitions of periodontitis was developed and suggested based on cumulative studies for almost 20 years [20]. In the workshop, three obviously different forms of periodontitis based on pathophysiology were categorized: necrotizing periodontitis, periodontitis as a direct manifestation of systemic disease, and periodontitis. Herein, we included the latter form. The periodontitis stage increased according to the severity, complexity, extent and distribution of the disease. Our prediction models for periodontitis showed the high accuracy based on this new classification system by using limited amounts of significant biomarkers.

In this study, we included all forms of MMP-8. Several studies found that the active forms of MMP could distinguish periodontitis [21,22]; however, in this study, all forms were significantly different. The increase was considered to reflect the increased leakage of all types of MMP-8 according to the severity of periodontitis. Interestingly, CRP did not show a significant difference, which was not consistent with other studies [23,24]. This might be because the detective capacity of ELISA is not sensitive enough to detect the changes in level of CRP [25,26]. Therefore, to monitor the level of CRP, more sensitive techniques should be used.

Our findings suggest that whole saliva can be used as a prospective diagnostic tool for periodontitis. Additionally, the proper selection of biomarkers in whole saliva was important in order to increase the sensitivity and specificity of the diagnosis of periodontitis [27,28]. On the other hand, this method had limitations in the early diagnosis with stage I. Other studies also showed similar limitations where the screening test could not distinguish early stage periodontitis [29]. By definition, stage I periodontitis is a bridge between gingivitis and periodontitis; thus, it can show lower levels of biomarkers than the severe forms. Other researchers tried to discriminate gingivitis and periodontitis through macrophage inflammatory protein-1α [30,31]. To overcome the problem, it was necessary to find additional biomarkers or develop more sensitive detecting techniques for early detection.

The diagnosis of periodontitis with clinical parameters is a very effective tool but it is time-consuming and labor intensive. The evaluation of clinical parameters is somewhat difficult to standardize and cannot monitor the real-time changes in periodontal disease progression [27,28]. Therefore, the method of whole saliva analysis could be an easier and simpler diagnostic tool for the

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detection of periodontitis. Additionally, salivary biomarkers can be a very prospective screening tool when one considers the high correlations between periodontitis and systemic disease [21,32,33].

Many researchers have reported the efficacy of GCF as a diagnostic method for chronic periodontitis [34-36]. However, most of the sampling methods cannot be obtained quantitatively; therefore, their reliability is considered low. At first, we tried but failed to obtain GCF quantitatively because of the high viscosity of GCF. Therefore, to measure GCF quantitatively, it is necessary to overcome these technical problems.

5. Conclusions

This study can contribute to screening for chronic periodontitis based on salivary biomarkers. The PA-I and PA-II model using microbial profiles and cytokines showed an improved tool for the diagnosis of periodontitis with whole saliva.

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