



The potentiality of salivary peptide biomarkers for screening patients with periodontal diseases by mass spectrometry

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ARTICLE INFO

Keywords:

Periodontitis

Gingivitis

Salivary biomarkers

Mass spectrometry

ABSTRACT

Background: Certain candidate biomarkers for periodontal diseases in saliva, gingival crevicular fluid (GCF), and serum were reported by some previous studies, but little evidence was obtained in their potentiality for screening patients with periodontal diseases.

Methods: Unstimulated whole saliva, GCF, and serum samples, which were collected from 17 patients with chronic periodontitis, 17 with gingivitis, and 16 periodontally healthy persons as control, were analysed by MALDI-TOF MS. Cluster analysis and receiver operating characteristic (ROC) curve analysis were carried out to evaluate the ability of candidate peptides to distinguish patients with periodontal diseases from healthy subjects. Nano-LC/ESI-MS/MS was performed to identify possible proteins that these peptides might derive from.

Results: Most of the differentially expressed peptides exhibited an increase in participants with chronic periodontitis and gingivitis compared with healthy controls. Cluster analysis showed a good clustering capacity between chronic periodontitis and healthy controls. Most AUCs for differentially expressed peptides were > 0.7, whereas some peptides from GCF and serum even exhibited AUCs of 0.9–1.0.

Conclusions: Some peptides in saliva, GCF, and serum act as biomarkers for chronic periodontitis and gingivitis, which have certain potentiality for screening patients with periodontal diseases and distinguishing them from healthy individuals in a comparatively large population by mass spectrometry.

1. Introduction

Periodontal diseases contain a wide range of inflammatory conditions that affect tooth-supporting structures, which could finally lead to tooth loss [1]. It is one of the most common chronic diseases suffered by people worldwide, with a global prevalence of 10.79% in 2010 [2]. Periodontal disease begins with gingivitis that is initiated by bacteria in dental plaque, which, if left untreated, may lead to chronic periodontitis [1]. Chronic periodontitis has been shown to have a significant impact on an individual's daily activities, such as negative effects on speech, nutrition, quality of life, and self-esteem [3,4]. Moreover, several studies have verified associations between chronic periodontitis and general diseases, such as cardiovascular disorders [5] and diabetes

[6]. Thus, chronic periodontitis is not just a threat to oral health, but also has an effect on systematic health.

Traditional diagnosis of periodontal diseases is still performed using clinical tests and tools, specifically measuring periodontal pocket depth, clinical attachment loss, radiographic alveolar bone level, bleeding on probing, and oral hygiene performance, which requires technically trained professionals [7,8]. However, these methods can only evaluate damage caused by previous destruction episodes, resulting in a retrospective diagnosis and lacking a reliable and objective approach to diagnosis and predict patients with periodontal diseases [7,9]. Furthermore, it is relatively difficult for the traditional diagnostic method to be used in large-scale screening. Therefore, a more efficient screening tool of patients at early stage of the disease is essential for both oral and

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<https://doi.org/10.1016/j.cca.2019.04.076>

Received 15 December 2018; Received in revised form 22 March 2019; Accepted 22 April 2019

Available online 23 April 2019

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general health [10,11]. Recent studies have attempted to identify a more rapid and reliable method that can screen and predict periodontal diseases based on saliva, gingival crevicular fluid (GCF), and serum biomarkers [12–14].

Serum, as a sensitive body fluid for systemic inflammatory response, has been studied to screen patients with periodontal diseases. Tabeta et al. [14] found that proprotein convertase subtilisin/kexin type 9 and high sensitivity C-reactive protein were increased and total bilirubin was decreased in chronic periodontitis patients compared with healthy controls. However, the application of serum is often limited because of the invasiveness of procedure and poor compliance. In addition, its components are susceptible to other infections and diseases. Gingival crevicular fluid (GCF) exists as a serum transudate, changing into an inflammatory exudate with the inflammatory events progress [15]. GCF collection and analysis have long been a popular approach to investigate localised inflammatory processes in periodontal diseases [16]. Recent studies have found that myeloid-related protein (MRP) 8/14 and matrix metalloproteinase (MMP) 8/13/14 were increased in GCF of patients with chronic periodontitis [17,18]. Although GCF is considered to mirror the periodontal health state of an individual, it also has certain disadvantages. For instance, GCF is produced in only microliter volumes, making its collection technically more challenging. It is prone to suffer from blood, saliva, or bacterial plaque contamination during paper strip collection, whilst the sampling procedure could produce some uncomfortable feelings [19,20]. Saliva, composed of secretions from major salivary glands comprising the parotid, submandibular, and sublingual glands, minor salivary gland secretions, and GCF, changes constantly in response to physiological regulatory processes and thus reflects the biochemical state of the body [13,21]. It is one of the main body fluids used to screen protein profiles of periodontal diseases, and it can not only reflect the local oral microenvironment but also reflect the systemic environment related to health status. A recent study found that salivary interleukin-1 β , interleukin-6, and MMP-8 were elevated in periodontitis patients compared with both healthy subjects and those with gingivitis [22]. Saliva collection is generally non-invasive, painless and technically simple, eliminating the need for trained professionals, and has lowest biological risks. In addition, saliva collection has higher safety, better applicability for large-scale populations, and higher co-operation from anxious patients, handicapped, children, or elderly people compared with GCF and serum collection [13,23].

Although previous studies that analysed serum, GCF, or salivary biomarkers to distinguish between periodontal diseases and a healthy state have been performed, few studies have utilized matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to analyse changes in salivary, GCF, and serum peptide profiles of patients with chronic periodontitis and gingivitis. Therefore, the aim of this study is to detect peptide biomarkers for discrimination between patients with periodontal diseases and healthy individuals. We also sought to explore the rapid screening potential of salivary peptides in periodontal diseases using MALDI-TOF MS and provide a reference for future large-scale screenings and early detection of patients with periodontal diseases.

2. Materials and methods

2.1. Ethics approval and informed consent

This study was ethically approved by the Peking University Biomedical Ethics Committee (issuing number: IRB00001052-16072) and carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). All participants provided written informed consent to take part in the study.

2.2. Subject selection

The study population was randomly selected from patients seeking

treatment at Peking University School of Stomatology from March to September 2017. A total of 50 individuals were involved in our study, including 17 patients with chronic periodontitis, 17 with gingivitis, and 16 periodontally healthy participants as control (Supplementary Table 1), who were also inter-group matched in gender and age as far as possible before the study commenced.

Inclusion criteria for all subjects consisted of: 1) older than 20 years, 2) had at least 20 naturally remaining teeth (excluding third molars), and 3) systemically healthy. Individuals were excluded from the study if they: 1) received periodontal treatment in the last 12 months, 2) used antibiotics or immunosuppressant medication within 3 months, 3) were pregnant or lactating, 4) were current or former smokers, 5) were diagnosed with systemic disease or disease/infection that may affect the periodontal health status (e.g. diabetes), 6) were wearing orthodontic appliances, 7) had oral mucosal inflammations, or 8) had serious untreated dental caries.

2.3. Clinical examination

Clinical examinations of all participants were performed by one specialised dentist using manual periodontal probes (PCPUNC 15; Hu-Friedy Mfg. Co., Inc., Chicago, IL, USA). The full mouth plaque scores of subjects were recorded. Clinical parameters, including clinical attachment loss (CAL), probing depth (PD), and bleeding on probing (BOP), were measured at six sites per tooth (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual, and disto-lingual). PD and CAL were measured to the nearest scale. Participants were classified into the group of chronic periodontitis (CP), gingivitis (G) and periodontally healthy control (H) based on their periodontal status according to diagnostic criteria proposed by the 1999 International Workshop for a Classification of Periodontal Diseases and Conditions [24]. The CP group ($n = 17$) included individuals who had $CAL \geq 1$ mm, $PD \geq 4$ mm, radiographic alveolar bone loss, and $> 30\%$ of teeth involved. The G group ($n = 17$) exhibited no CAL, no site with $PD > 3$ mm, $BOP > 20\%$, and no radiographic alveolar bone loss. The H group ($n = 16$) exhibited no sites with attachment loss, no sites with $PD > 3$ mm, $BOP \leq 20\%$, and no radiographic alveolar bone loss.

2.4. Saliva collection and processing

All participants were instructed not to use any oral hygiene practices, including toothbrushing, flossing, mouth rinsing, and gum chewing, for at least 2 h until sampling. Before collection, every subject was asked to rinse their oral cavity with water and then rested for 10 min. Unstimulated whole saliva was collected at 8:00–9:00 a.m. for 10 min with the assistance of one researcher, who would help all the subjects one after another in sampling procedures. Samples were immediately placed on ice. Then, 2 mL saliva aliquot of each sample was centrifuged with the speed of $10,000 \times g$ for 10 min at $4^\circ C$ to remove insoluble material, cells, and debris. Supernatants were collected and supplemented with a proteinase inhibitor combination of 1 mM ethylenediaminetetraacetic acid (Sigma-Aldrich, St. Louis, MO, USA) and 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich). All samples were stored at $-80^\circ C$ until further analysis.

2.5. Gingival crevicular fluid collection and processing

GCF samples were collected from all participants on mesio-buccal and disto-buccal sites of the same six sampling teeth (11, 31, 16, 26, 36, and 46). Before GCF sampling, the supragingival plaque was removed with a sterile curette, and surfaces were gently dried by air syringe and isolated by cotton rolls. Twelve paper strips (Whatman 1 MM chromatography paper, Whatman Lab Sales Ltd., Kent, England) were introduced into the gingival crevice for 30 s. These paper strips were immediately deposited into a tube containing 350 μL phosphate-buffered saline and then placed on ice. Supernatants were collected after

centrifuging with the speed of $10,000 \times g$ for 10 min at 4 °C. All samples were stored at –80 °C until further analysis.

2.6. Serum collection and processing

All individuals were refrained from food and drinking intake for 8 h prior to blood sampling. 5 mL whole blood sample was collected from each subject by a professional inspector. Collected samples were allowed to clot at room temperature for 4 h, then the precipitates were discarded after stratification, with the supernatants retained and kept at –80 °C until further analysis.

2.7. WCX fractionation and MALDI-TOF MS

All samples were fractionated using a weak cation exchange magnetic bead (WCX MB) kit (Bioyong Tech, Beijing, China). Samples were isolated and purified by the following steps: 1) 150 μ L of MB-WCX binding solution, 20 μ L of beads, and 10 μ L of sample were mixed carefully and incubated for 5 min at room temperature. 2) The tubes were placed on the MB separation device for 1 min to collect the beads onto the tube wall, and then the supernatant was removed. 3) The beads were washed by 150 μ L washing solution. 2 min later, the tubes were placed on the separation device for 1 min. 4) Step 3 was repeated and all the supernatant was removed. 5) 10 μ L of MB elution solution was added, and the beads were allowed to gather on the tube wall in the separation device for 2 min. 6) The clear supernatant was transferred to a new tube, and the peptides were analysed immediately on a ClinTOF instrument (Bioyong Technologies Inc.) or stored at –20 °C and analysed within 24 h.

The matrix solution was 8 mg/mL CHCA in 50% acetonitrile/0.1% TFA/49.9% deionized water. First, 1 μ L of the purified peptide solution was spotted onto a MALDI-TOF MS target and then dried at room temperature. Then 1 μ L of the matrix solution was spotted to cover the sample and then dried again. MALDI-TOF MS was conducted using a ClinTOF instrument (Bioyong Tech). Before analysis, we employed a three-peptide mixture (monoisotopic molecular weights of 1533.8582, 2465.1989, and 5730.6087 Da; Sigma product numbers P2613, A8346 and I6279, respectively) to calibrate the mass spectrometry. Profile spectra were obtained from an average of 400 laser shots per sample. The m/z in the range of 1000–10,000 Da was collected. The experiments were performed for three times to acquire the mean values of m/z values and intensities of the peaks. The above methodology has been successfully utilized in previous studies by our research group, which reliability was validated [25,26].

2.8. Data processing and statistical analysis

All the spectra obtained from the samples were analysed by BioExplorer 1.0 (Bioyong Tech) to acquire the mean relative peak intensities, subtract chemical and electrical noises, normalize spectra using total ion current, and determine intensities and peak mass-to-charge ratio (m/z) values in the range of 1000–10,000 Da. A signal-to-noise (S/N) ratio > 5 was required. To align the spectra, a mass shift of no $> 0.1\%$ was determined. The peak area was analysed for quantitative standardization.

All the experiments were repeated for three times. Data were analysed using the BioExplorer 1.0 statistical package. Differences in peptide levels amongst samples between selected groups were evaluated using Student's t -tests or the Wilcoxon rank sum test according to the results of normality tests. Differences in age and gender were compared using t -test and χ^2 test. Cluster analysis was carried out using HemI (Heatmap Illustrator, version 1.0). Receiver operating characteristic (ROC) curve analysis of differentially expressed peptides was performed using SPSS Statistics 22.0 software (IBM, Armonk, NY, USA). $P < .05$ was considered to indicate statistical significance and all P values were two-sided.

2.9. Identification of peptides by nano-liquid chromatography-electrospray ionization-tandem mass spectrometry (nano-LC/ESI-MS/MS)

Nano-LC/ESI-MS/MS was performed to identify the sequences of candidate peptides. The supernatants were processed by WCXMB as described above and then centrifuged with the speed of $10,000 \times g$ for 20 min at 4 °C to remove impurities. The supernatants were purified using a 0.22 μ m filter. Each sample was separated by nano-UPLC system EASY-nLC1000 (Thermo Fisher Scientific), and the flow rate of the equilibrated BEH nano ACQUITY column 100 $\times$ 100 mm was 400 μ L/min. Q-Exactive mass spectrometer (Thermo Fisher Scientific) was then used to perform tandem mass spectrometry. The voltage of the ion source was 3.5 kV, the analysis time was set to 120 min, and higher-energy collision dissociation (HCD) was performed to fragment the peptides. By gathering twenty fragmented fingerprints after each single full scan, the m/z values of the peptide fragments were acquired. At m/z 200, survey scans were obtained at a resolution of 70,000 and resolution for HCD spectra was set to 17,500. The initial screening was carried out after inputting the MS/MS figure to the PD software (Proteome Discoverer 1.4, Thermo Fisher Scientific).

The parameters used were listed below: the minimum number of peaks in MS/MS figures was set as 10, the range of molecular weight of the parent ion was 350–6000 Da, and the thresholds of S/N ratio was 1.5. After initial screening, the mass spectra were searched using Mascot software (version: 2.3.2) based on variable modification and fixed modification. Database NCBI nr 20120419 (17893860 sequences, 6141683785 residues) and Percolator method (false discovery rate [FDR] $\leq 1\%$, accuracy of peptide tol: 20 ppm) was used for data analysis.

3. Results

3.1. Characteristics of study subjects

The CP group included 17 patients with chronic periodontitis (mean age 40.12 ± 11.60 years, 8 males/9 females), The G group included 17 patients with gingivitis (mean age 34.29 ± 10.72 years, 6 males/11 females), and the H group included 16 periodontally healthy individuals as controls (mean age 33.06 ± 11.30 years, 5 males/11 females). There were no significant differences in the mean age (CP vs. H: $P = .087$; G vs. H: $P = .750$) and sex proportion (CP vs. H: $P = .353$; G vs. H: $P = .805$) between selected groups.

3.2. Differentially expressed peptide peaks

Saliva, GCF, and serum samples from the three groups were analysed by MALDI-TOF MS, and peptide fingerprints were obtained in the range of 1000–10,000 Da (Fig. 1).

In the comparison between CP and H groups, 91 salivary peptide peaks were detected, 7 of which were significantly different between the two groups. Two peptide peaks (m/z values: 1836.4 Da and 1858.4 Da) had lower level of intensities in the CP group, while the rest of differentially expressed peptides (m/z values: 1044.0 Da, 1122.0 Da, 1147.1 Da, 1583.9 Da and 3434.4 Da; Fig. 2a) had higher level of intensities. 58 peptide peaks were detected in serum, 13 of which showed significant difference between the CP and H groups. Amongst the differentially expressed peptides in serum, those with m/z values of 1075.6 Da and 1122.0 Da exhibited at a lower level in patients with chronic periodontitis compared with healthy individuals. In contrast, peptide peaks with m/z values at 1940.3 Da, 2077.1 Da, 2654.3 Da, 2946.3 Da, 2984.0 Da, 3233.9 Da, 3874.9 Da, 4635.2 Da, 5893.1 Da, 7751.9 Da and 9272.8 Da were at a higher level in the CP group (Fig. 2b). 48 peptide peaks were detected in GCF, amongst which four were significantly higher in CP group compared with healthy controls (m/z values: 3434.4 Da, 4126.6 Da, 5407.7 Da and 5416.0 Da; Fig. 2c).

In total 113 salivary peptide peaks were detected in patients with

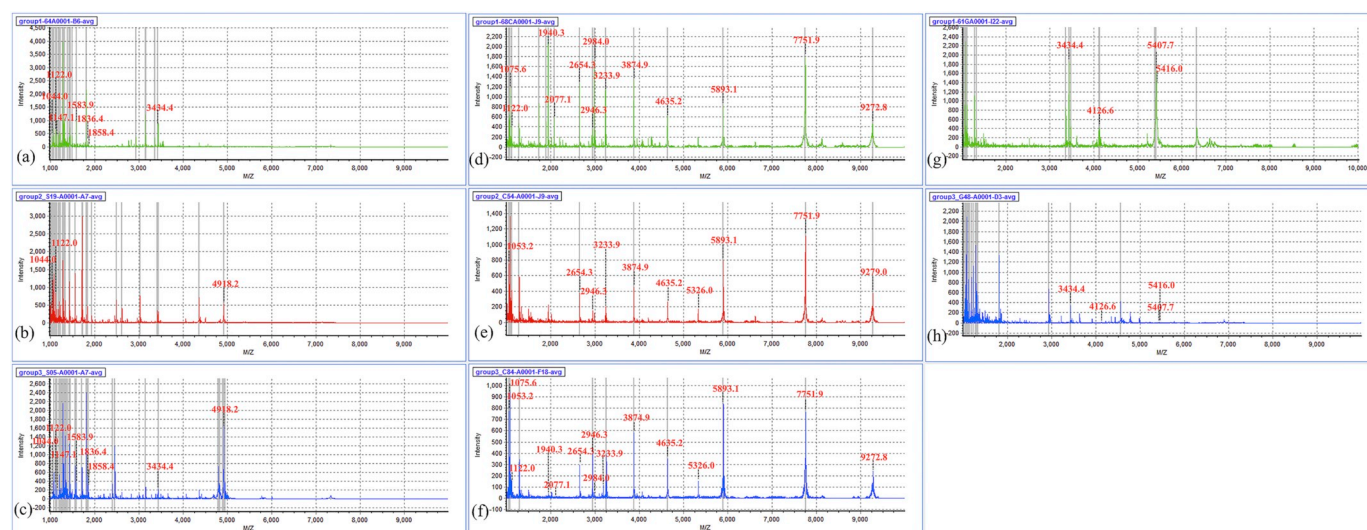


Fig. 1. Peptide fingerprints of representative participants in the range of 1000–10,000 Da m/z . (a–c) peptide mass spectra of saliva; (d–f) peptide mass spectra of serum; (g–h) peptide mass spectra of GCF (a/d/g. a representative patient with chronic periodontitis, b/e. a representative patient with gingivitis, c/f/h. a representative participant as healthy control). The differentially expressed peptide peaks were labelled ($P < .05$). m/z , mass-to-charge ratio.

gingivitis, amongst which three were significantly different from healthy controls. Intensities of peptides with m/z values of 1044.0 Da and 1122.0 Da were higher in patients with gingivitis, whilst those of peptide peaks of 4918.2 Da were lower in the patients (Fig. 2d). 58 serum peptide peaks were detected, amongst which 10 exhibited significantly higher level of intensities in patients with gingivitis (m/z values: 1053.2 Da, 2654.3 Da, 2946.3 Da, 3233.9 Da, 3874.9 Da, 4635.2 Da, 5326.0 Da, 5893.1 Da, 7751.9 Da and 9279.0 Da; Fig. 2e).

3.3. Cluster analysis of differentially expressed peptides

Hierarchical cluster analysis was performed based on the intensities of differentially expressed peptide peaks of each participant. We observed that differentially expressed peptides from saliva and GCF had

relatively good clustering capacity and differentially expressed serum peptides had reasonably good clustering capacity to distinguish patients with chronic periodontitis from healthy subjects (Fig. 3a–c). However, for gingivitis all differentially expressed peptides from saliva and serum presented relatively poorer clustering capability (Fig. 3d–e).

3.4. Distinguishing ability of differentially expressed peptides between subjects with periodontal diseases and healthy controls

Receiver operating characteristic (ROC) curve analysis was performed using SPSS Statistics 22.0 software (IBM). Area under the curve (AUC) values of differentially expressed peptide peaks, as an evaluating criteria of the probability to distinguish subjects with periodontal diseases from healthy controls, were computed.

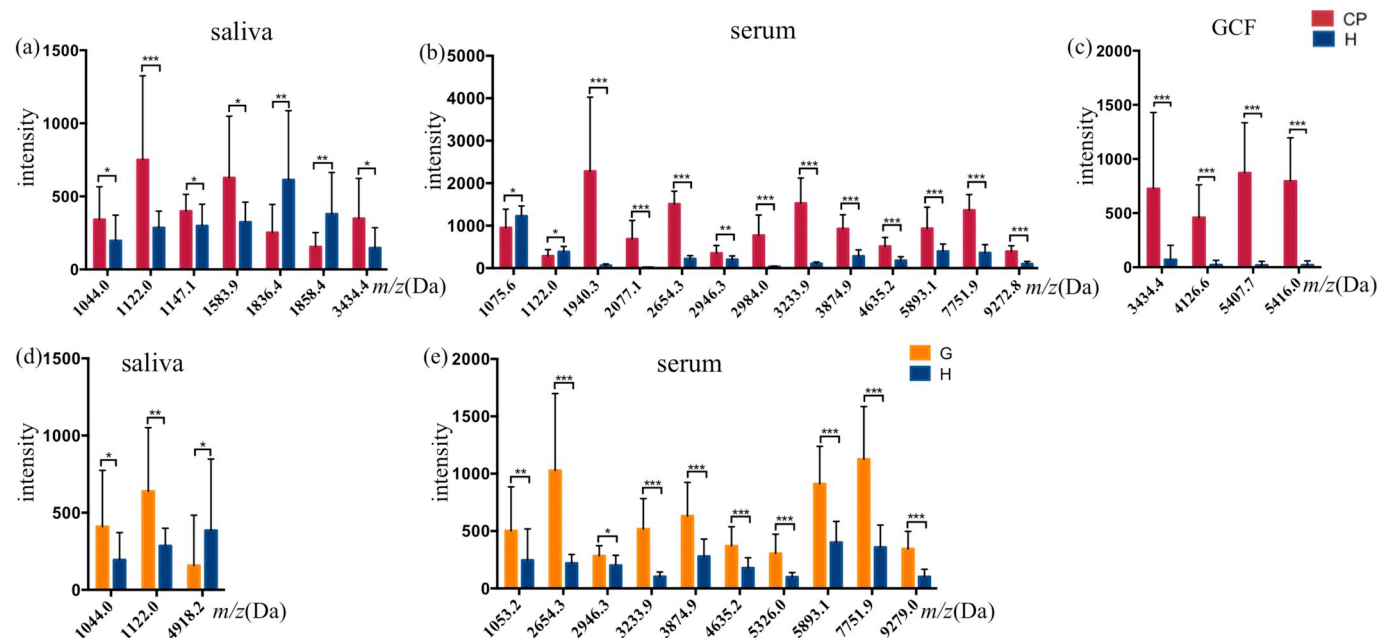


Fig. 2. Histogram of differentially expressed peptide peaks in selected groups. (a–c) Differentially expressed peptide peaks between the CP and H groups (a. saliva, b. serum, c. GCF); (d–e) Differentially expressed peptide peaks between the G and H groups (d. saliva, e. serum). Most of the differentially expressed peptide peaks were increased in the CP and G groups, except for salivary peptide peaks with m/z value of 1836.4, 1858.4 and 4918.2 Da, and serum peptide peaks at 1075.6 and 1122.0 Da. * $P < .05$, ** $P < .01$, *** $P < .001$.

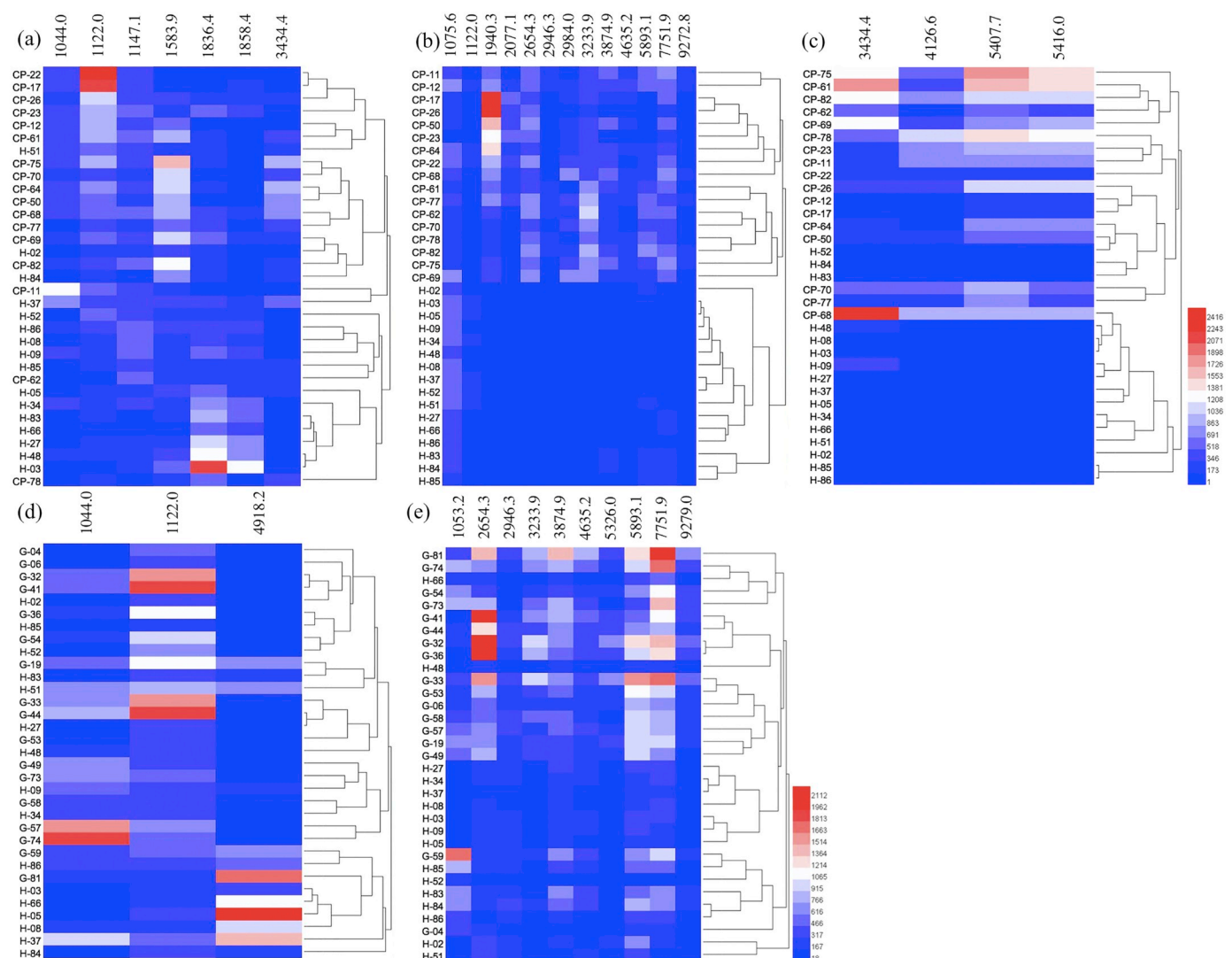


Fig. 3. Cluster analysis of all the differentially expressed peptide peaks. Rows represent samples, whilst columns showed m/z peaks by the average molecular weight. In the row labels, numbers behind “-” represents the ID of subjects. (a–c) Cluster analysis of differentially peptides between the CP and H groups (a. saliva, b. serum, c. GCF); (d–e) Cluster analysis of differentially peptides between the G and H groups (d. saliva, e. serum).

Fig. 4 shows the ROC curves for differentially expressed peptide peaks. The ability of these peptides to predict chronic periodontitis is shown in Table 1. The AUCs of these peptide peaks in saliva ranged from 0.688 to 0.860, whereas the AUC for the “sum” value, which represents the change in all salivary differentially expressed peptides, was 0.897. The AUCs for differentially expressed peptide peaks in GCF ranged from 0.926 to 1.000. For peptide peaks in serum, the AUCs ranged from 0.695 to 1.000.

In the G and H groups, AUCs of peptide peaks in saliva ranged from 0.728 to 0.829, and the AUC for the “sum” value was 0.849. For peptide peaks in serum, AUCs ranged from 0.774 to 0.978 (Table 2).

3.5. Identification of candidate peptides

Nano-LC/ESI-MS/MS analysis was carried out to identify possible proteins which these peptides may derive from. If the difference between the experimental and theoretical m/z values was less than one-thousandth molecular weight, then we considered them to be the same peptide. We successfully identified the peptide with experimental m/z value of 1075.6 Da to be derived from serum albumin, and that of 1122.0 Da to be a segment of immunoglobulin kappa variable 4–1, whereas other three peptides (experimental m/z values: 1147.1 Da,

3434.4 Da and 3874.9 Da) were identified to be haptoglobin (Table 3).

4. Discussion

Chronic periodontitis is one of the most common diseases worldwide, and gingivitis is the “gateway” to periodontitis for a significant portion of the population [15]. Chronic periodontitis, if not controlled, will probably not only impact oral health but also contribute to adverse effects on systemic health [27]. MALDI-TOF MS is a high-throughput, rapid, inexpensive, and reliable technique with high sensitivity and resolution, and it has been used to analyse peptides in many oral and systemic diseases, such as chronic periodontitis, oral cancer [28], Sjögren's syndrome [29], ovarian cancer [30], and lung adenocarcinoma [31]. To our knowledge, this is one of the few studies collecting saliva, GCF, and serum to evaluate changes in peptide profiles of patients with chronic periodontitis and gingivitis by MALDI-TOF MS.

In this study, we found 7 differentially expressed peptide peaks in saliva, 4 in GCF, and 13 in serum between patients with chronic periodontitis and healthy controls. Besides, 3 differentially expressed peptide peaks in saliva and 10 in serum were detected between patients with gingivitis and healthy controls. Most of these peptide peaks were at a higher level in patients with chronic periodontitis and gingivitis,

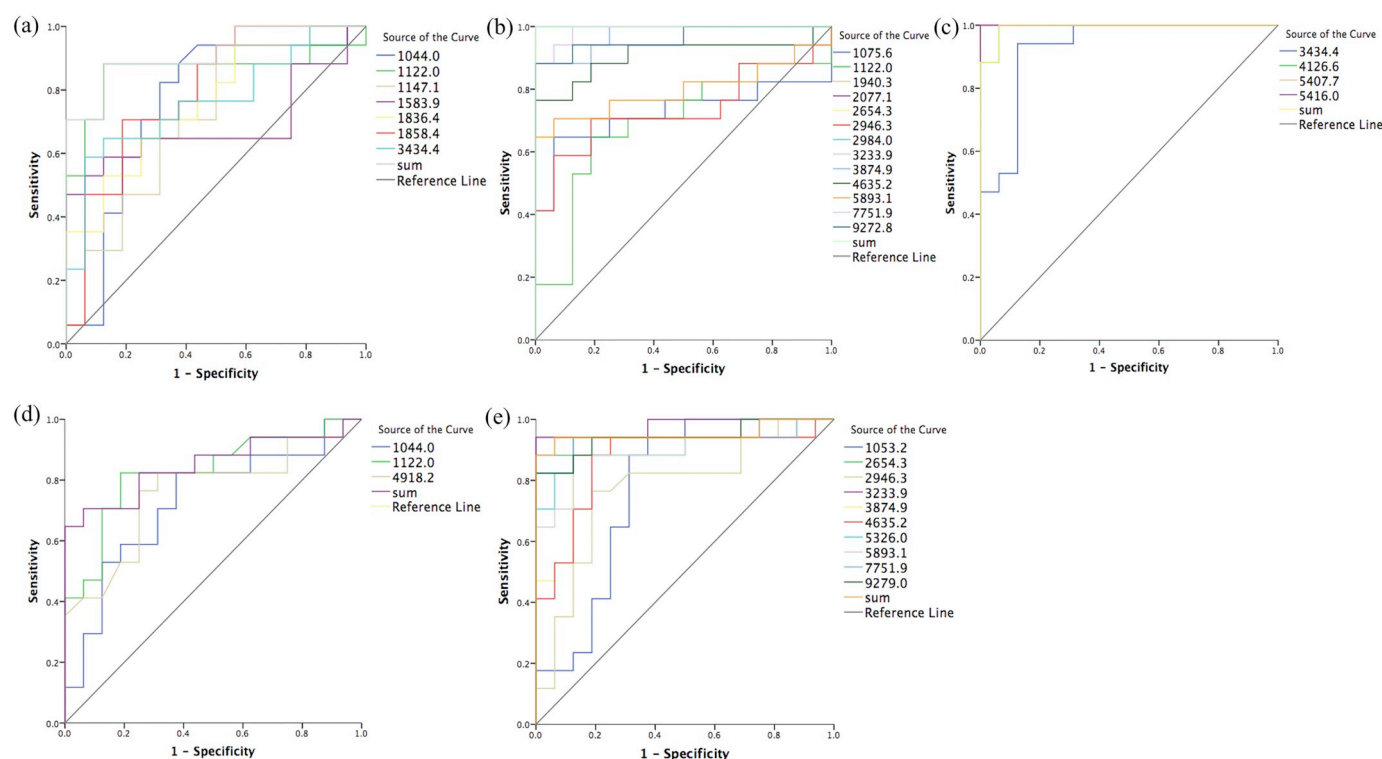


Fig. 4. Receiver operating characteristic (ROC) analysis of differentially expressed peptide peaks. (a–c) Comparison between the CP and H groups (a. saliva, b. serum, c. GCF). (d–e) Comparison between the G and H groups (d. saliva, e. serum).

Table 1

The area under receiver operating characteristic curve of differentially expressed peptide peaks between the CP and H groups.

Sample	Peptide peak (m/z)	AUC	P value	95% CI
Saliva	1044.0	0.756	0.012	0.576–0.935
	1122.0	0.860	< 0.001	0.715–1.000
	1147.1	0.706	0.044	0.525–0.886
	1583.9	0.688	0.066	0.493–0.882
	1836.4	0.776	0.007	0.619–0.933
	1858.4	0.794	0.004	0.637–0.951
	3434.4	0.765	0.009	0.599–0.930
	sum	0.897	< 0.001	0.790–1.000
	sum	0.897	< 0.001	0.790–1.000
GCF	3434.4	0.926	< 0.001	0.835–1.000
	4126.6	0.993	< 0.001	0.973–1.000
	5407.7	1.000	< 0.001	1.000–1.000
	5416.0	1.000	< 0.001	1.000–1.000
	sum	0.993	< 0.001	0.973–1.000
Serum	1075.6	0.724	0.028	0.532–0.916
	1122.0	0.695	0.056	0.507–0.883
	1940.3	1.000	< 0.001	1.000–1.000
	2077.1	1.000	< 0.001	1.000–1.000
	2654.3	1.000	< 0.001	1.000–1.000
	2946.3	0.735	0.021	0.554–0.917
	2984.0	1.000	< 0.001	1.000–1.000
	3233.9	1.000	< 0.001	1.000–1.000
	3874.9	0.974	< 0.001	0.930–1.000
	4635.2	0.908	< 0.001	0.793–1.000
	5893.1	0.798	0.004	0.630–0.966
	7751.9	0.989	< 0.001	0.965–1.000
	9272.8	0.963	< 0.001	0.901–1.000
	sum	1.000	< 0.001	1.000–1.000

Footnotes: “sum”, the summary value of intensity of the peaks that exhibited an increase in the CP group minus those who exhibited a decrease.

except for those with m/z at 1836.4 Da, 1858.4 Da and 4918.2 Da in saliva, and 1075.6 Da and 1122.0 Da in serum (Fig. 2).

With nano-LC/ESI-MS/MS analysis, we successfully identified

Table 2

The area under receiver operating characteristic curve of differentially expressed peptide peaks between the G and H groups.

Sample	Peptide peak (m/z)	AUC	P value	95% CI
Saliva	1044.0	0.728	0.026	0.551–0.905
	1122.0	0.829	0.001	0.685–0.973
	4918.2	0.759	0.011	0.590–0.928
	sum	0.849	0.001	0.711–0.988
	sum	0.849	0.001	0.711–0.988
Serum	1053.2	0.776	0.007	0.605–0.946
	2654.3	0.949	< 0.001	0.861–1.000
	2946.3	0.774	0.007	0.607–0.941
	3233.9	0.978	< 0.001	0.932–1.000
	3874.9	0.886	< 0.001	0.763–1.000
	4635.2	0.868	< 0.001	0.735–1.000
	5326.0	0.912	< 0.001	0.806–1.000
	5893.1	0.904	< 0.001	0.799–1.000
	7751.9	0.934	< 0.001	0.832–1.000
	9279.0	0.941	< 0.001	0.856–1.000
	sum	0.952	< 0.001	0.866–1.000

Footnotes: “sum”, the summary value of intensity of the peaks that exhibited an increase in the G group minus those who exhibited a decrease.

peptides with m/z value of 1122.0 Da to be immunoglobulin kappa variable 4–1, which is the V segment of the variable domain of immunoglobulin light chain that participates in antigen recognition [32]. The level of this protein was higher in saliva of both patients with periodontitis and those with gingivitis, exhibiting the human defending response to antigens. This is rarely reported in other studies. Interestingly, immunoglobulin kappa variable 4–1 was at a lower level in serum of patients with chronic periodontitis. This might be attributed to the influence of some potentially underlying infections and diseases, which has manifestations in the serum. Although the participants included in this study were reported by themselves to be systemically healthy, their general health status is still in doubt. Further research is needed to explore the probable reasons for this phenomenon.

Table 3
Identification of candidate peptide biomarkers for periodontal diseases.

Experimental <i>m/z</i> value	Theoretical <i>m/z</i> value	Tendency in CP/G group			Protein name	Peptide sequence
		Saliva	GCF	Serum		
1075.6	1074.5	—	—	↓	Serum albumin	LDELRDEGK
1122.0	1122.6	↑	—	↓	Immunoglobulin kappa variable 4-1	LLIYWASTR
1147.1	1146.5	↑	—	—	Haptoglobin	HYEGSTVPEK
3434.4	3432.6	↑	↑	—	Haptoglobin	AVGDKLPECEADDGCPKPPEIAHGYVEHSVR
3874.9	3874.7	—	—	↑	Haptoglobin	YQEDTCYGDAGSAFAVHDLLEDTWYATGILSFDK

We identified peptide of 3874.9 Da in serum, 1147.1 Da and 3434.4 Da in saliva, and 3434.4 Da in GCF to be derived from haptoglobin (Hp), which exhibited a higher level in patients with gingivitis and chronic periodontitis. Previous studies had also reported elevated levels of Hp in saliva, serum and GCF in individuals with periodontitis [33–35]. Hp is an acute-phase protein, which exerts a broad range of anti-inflammation activities and could act indirectly as an antioxidant and bacteriostatic agent [36]. Its expression increases in response to injury or infection [37]. By propagating various anti-inflammation activities, Hp contributes to the recovery of homeostasis after systemic or local infection [36]. Thus, the up-regulation of haptoglobin levels in this study may reflect anti-inflammatory and reestablishment process of the periodontium.

Peptide of 1075.6 Da in serum was identified to be serum albumin, which had a lower level of intensity in the CP group. Human serum albumin regulates both colloid osmotic pressure and capillary membrane permeability, ligand binding and transport, and free radical scavenging, which has antioxidant and circulatory protective properties [38]. The mechanism of relationships between serum albumin and periodontal diseases is not well understood. Previous studies hypothesised that the decline of serum albumin in patients with periodontitis was related to nutritional deficiencies [39,40]. Some other researchers believed that changes in nutrient intake had little effects on the level of serum albumin, but inflammation was a more reasonable explanation [41–43]. According to literatures, interleukin-1 (IL-1) is an important mediator of the inflammatory response, which will weaken the synthesis of albumin under the condition of inflammation [44] and has been reported to be at a higher level in patients with periodontitis [22,45,46]. This manifestation of elevated inflammatory factors caused by periodontal infections might contribute to the lower level of serum albumin in the CP group. Since subjects included in this study were systematically healthy and the majority of them had mild to moderate periodontitis with a comparatively lower number of missing teeth, impaired nutritional status might be an unfavourable reason for this point.

Cluster analysis showed that the differentially expressed peptide peaks in saliva and serum had a better classifying ability of the CP and H groups than that of the G and H groups. Since periodontitis is a more serious and deeper inflammation than gingivitis, a larger difference might exist between patients with periodontitis and periodontally healthy individuals. Additionally, differentially expressed peptide peaks in serum showed better clustering ability than that in saliva and GCF when classifying patients with chronic periodontitis and healthy subjects (Fig. 3).

AUC is a measure of how well the model distinguishes disease from healthy control. Most of the differentially expressed peptide peaks had AUC above 0.7, but AUC for peptides of 1122.0 Da in serum and 1583.9 Da in saliva were below 0.7 ($P > .05$), demonstrating that though these two peptides were significantly different between selected groups, their diagnostic significance was not strong enough. To distinguish patients with gingivitis and the healthy controls, AUCs ranged from 0.728 to 0.829 (Table 2), which represented certain distinguishing ability. AUCs of differentially expressed salivary peptides between the CP and H groups ranged from 0.706 to 0.860 (Table 1). These peptides

may have the potentiality to be selected as candidate biomarkers for periodontal diseases, which is similar with some previous studies reported [22,47,48].

As for differentially expressed peptide peaks in serum and GCF, we found that peptides in serum of 2654.3 Da, 3233.9 Da, 5326.0 Da, 5893.1 Da, 7751.9 Da and 9279.0 Da had AUCs higher than 0.9 (Table 2). Some differentially expressed peptides in GCF and serum had AUCs higher than 0.9 and some even reached 1.0 when comparing the CP and H groups (*m/z* values: 3434.4 Da, 4126.6 Da, 5407.7 Da and 5416.0 Da in GCF; and 1940.3 Da, 2077.1 Da, 2654.3 Da, 2984.0 Da, 3233.9 Da, 3874.9 Da, 4635.2 Da, 7751.9 Da and 9272.8 Da in serum; Table 1), indicating more reliable distinguishing ability of GCF and serum than saliva. This finding is similar to that previously reported by Mauramo et al. [49], who found a higher AUC for MMP-8 in GCF (0.71) than that in saliva (0.67), and it might be explained by the fact that GCF is more specific to periodontal diseases than saliva, since it derives from local site of the actual inflammatory periodontal tissues [8]. On the contrary, some studies observed higher AUCs of biomarkers in saliva than that of GCF and serum to distinguish individuals with periodontal diseases and healthy controls [50,51], which may be attributed to the variant characteristics of saliva, serum and GCF. Saliva, as an oral biofluid, may have better ability in reflecting oral-related diseases than the serum. Moreover, GCF can only reflect the inflammatory status of certain specific sites, while saliva has elements that can reflect the activity of all periodontal sites, making it better reflective of the overall periodontal status [52].

Although some GCF and serum peptides in our study showed a better ability to distinguish patients with periodontal diseases from healthy controls, saliva still has undoubted potentiality in screening individuals with gingivitis and periodontitis. In order to improve the ability of saliva in distinguishing individuals with periodontal diseases and the controls, we calculated the “sum” value to represent the changes of all the differentially expressed peptide peaks. In the present study, the AUCs for the “sum” value in chronic periodontitis and gingivitis reached 0.897 and 0.849, respectively, showing relatively satisfactory distinguishing ability (Tables 1 and 2). Previous studies had also found that AUC would be improved by the combination of several biomarkers compared with one single biomarker [22,53]. Saliva collection can be non-invasive and dispensable of professional skills and training, which is safer for people to handle and offers a cost-effective approach for screening diseases in a comparatively large population [16,52]. Thus, salivary tests offer a rapid, simple, easy, and safe approach for disease detection. It is a “real-time” fluid that can indicate an individual's health status at the moment of collection [13], which could be prospective in diagnosis and surveillance of periodontal disease [54] and other oral diseases, such as dental caries [26] and lichen planus [55]. Moreover, salivary proteins contains a number of serum constituents, making it of important diagnostic values in systemic diseases, such as cardiovascular disease [56], diabetes mellitus [57], and lung cancer [58]. Considering the advantages of saliva and its ability to distinguish individuals with periodontal diseases from healthy controls, we believe it has the potentiality to be used as an appropriate tool for rapid screening for patients with periodontal diseases in a comparatively large population.

Some limitations of the present study should be noted. First, some peptides are difficult to be identified because of their dynamic transformation after secretion and the incompleteness of protein databases. Although we considered two peptides to be the same one when the difference between the experimental and theoretical m/z values was within one-thousandth of molecular weight, it remains possible that the two peptides might be derived from different proteins. Thus, further validation of the candidate peptides by comparison with the ever-updating database in the future could provide stronger evidence. Second, this is a cross-sectional study, which could not fully confirm the causal relationship between the peptide biomarkers and the disease, leaving further validation in a larger sample size required to demonstrate their diagnostic capabilities. Third, the comparison of differentially expressed peptides between selected groups may be affected by the processing procedures of GCF samples due to a lack of precise quantification before dilution in our study. Hence, we finally kept only four peptide peaks in GCF with a mean difference of intensity > 10 times higher in the CP group compared with the group of healthy controls, which was according to previous studies that the variation of the mean volume of GCF amongst individuals was no > 10 times [59,60]. We consider it would be reasonable that these four peptide peaks could still represent the difference of peptide abundance between the CP and H groups, as they all exhibited an intensity of 10 times higher in the CP group. However, since Preianò et al. found that different diluting ratios and storage conditions could lead to changes in the results of MALDI-TOF MS analysis of GCF [61,62], the quantification of GCF is indeed needed to make more precise comparisons.

The occurrence and development of periodontal diseases exhibited as a complex inflammatory process. It is expectable that the detection of changes of components in saliva, GCF, serum could reflect the host responses to inflammations, screen patients with periodontal diseases or individuals at a higher risk of this disease, and explore potential mechanisms of the pathological process by analysing the functions of candidate biomarkers. With the development of proteomic and peptidomic technologies and the improvement of protein databases, we believe that more comprehensive holistic analysis of inflammatory status and host responses could reveal more specific biomarkers that could be used for the prediction, diagnosis, and surveillance of periodontal diseases.

5. Conclusions

In this study, we found that some peptides in saliva, GCF, and serum act as biomarkers for chronic periodontitis and gingivitis, which have certain potentiality for screening patients with periodontal diseases and distinguishing them from healthy individuals in a comparatively large population by mass spectrometry.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cca.2019.04.076>.

Acknowledgements

This study was funded by the National Natural Science Foundation of China (grant number: 81801037), Peking University School and Hospital of Stomatology (grant number: PKUSS20170105), Beijing Municipal Science and Technology Commission (grant number: Z181100001618015), Ministry of Science and Technology of the People's Republic of China (grant number: 2018FY101005), Haidian District Health Commission, and the Joint International Research Centre of Translational and Clinical Research between Peking University Health Science Centre and King's College London.

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