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Peptidomic changes of saliva after non-surgical treatment of stage I/II generalized periodontitis

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Abstract

Objective: To explore the changes of peptidome profiles of saliva, serum, and gingival crevicular fluid (GCF) before and after non-surgical periodontal treatment in patients with generalized periodontitis (stage I/II).

Subjects and methods: Saliva, serum, and GCF samples were collected from 17 patients at baseline (T_0), one week after ultrasonic supragingival scaling (T_1) and eight weeks after subgingival scaling and root planning (T_2). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was carried out to detect changes in peptidomic profiles. Then, nano-liquid chromatography-electrospray ionization-tandem mass spectrometry (nano-LC/ESI-MS/MS) was performed to identify potential peptide biomarkers.

Results: Most of the peptides from the patients exhibited a decreasing trend from the time point of pretreatment to that of post-treatment. Cluster analysis and scatter plots using these peptides indicated that salivary peptidome has an acceptable capability of reflecting the status of stage I/II generalized periodontitis. Seven of these peptides were successfully identified as α-1-antitrypsin, immunoglobulin κ variable 4-1, haptoglobin, and immunoglobulin heavy constant γ2.

Conclusions: Certain peptides in saliva, serum, and GCF were down-regulated after non-surgical periodontal treatment, demonstrating the application prospects of saliva in monitoring and surveillance of periodontal diseases in both clinical settings and communities.

KEYWORDS

chronic periodontitis, mass spectrometry, non-surgical treatment, saliva

Chao Yuan and Zhangke Ma contributed equally to this work and share the first authorship.

1 | INTRODUCTION

Periodontitis is a serious irreversible disease characterized by periodontium destruction (Pihlstrom et al., 2005), the severe type of which was the sixth most prevalent condition worldwide in 2010 (Frencken et al., 2017). Notably, periodontitis occurred with a particularly high prevalence in older individuals with low incomes (Slots, 2017), leading to impaired dentitions and physical and psychological functions (Papapanou & Susin, 2017). Mechanical root cleaning as a treatment had been advocated for centuries, and scaling and root planning (SRP) was an established "gold standard" treatment for periodontitis (Dentino et al., 2013; Krishna & De Stefano, 2016). However, periodontitis was still the main cause of tooth loss, and tooth loss and chewing dysfunction could affect personal nutrition, quality of life, and self-esteem, causing large socioeconomic impacts and medical costs (Petersen & Ogawa, 2012; Pihlstrom et al., 2005). Furthermore, there was increasing evidence indicating that periodontal diseases were undermining oral health and meanwhile exerting impact, by oral biofilms, bacterial products, and inflammatory mediators, on general health, particularly for diabetes, atherosclerosis, rheumatoid arthritis, and pulmonary infections (Mealey, 1999; Seymour et al., 2007). Therefore, it is important to detect diseases early, monitor disease progression, and take targeted prevention and control measures. Since the current diagnosis and evaluation methods are mainly used for assessing past disease and previous inflammatory damage, and the multicomponent pathologies of periodontitis are typically complex, several studies and reviews have highlighted the applicable potential of saliva, gingival crevicular fluid (GCF), and serum acting as periodontal disease biomarkers that may facilitate the diagnosis or progression of the disease or efficacy of the treatment program (Bostanci & Belibasakis, 2018; Guzman et al., 2014; Lorenzo-Pouso et al., 2018; Tsuchida et al., 2018; Zhang et al., 2009).

The oral environment is unceasingly bathed by saliva and GCF, both of which are two significant physiological fluids. Saliva is a promising biofluid that has received the attention of researchers in recent years. It is widely accepted that saliva is a potential medium to evaluate individual health status for its easy accessibility and close relationship with serum. First, saliva can be collected easily, non-invasive, safe, relatively stress-free and cost-effective compared with other body fluids. Taking a saliva sample is less technically demanding because it only requires limited training without highly sophisticated staff. Second, salivary biomarkers may thoroughly demonstrate the status of the oral cavity as well as systemic health due to the close relationship between saliva and other body fluids (Pfaffe et al., 2011; Trindade et al., 2014). Similarly, GCF is also a large reservoir of molecular information, playing an important role in the transportation of various periodontal bacterial products or host-derived immune components (Bostanci & Belibasakis, 2018). However, GCF collection can be technique-sensitive and time-consuming, with increased possible contamination by gingival bleeding (Tsuchida et al., 2018). Considering the association between periodontitis and systemic health, the serum is also a valuable physiological fluid for the identification of diagnosis and prognosis-associated biomarkers in inflammatory diseases. For instance, the levels of complement component 3, complement factor H, and ceruloplasmin were significantly different in patients with both rheumatoid arthritis (RA) and CP compared with healthy controls and patients with RA or CP only (Yokoyama et al., 2014).

Among studies aimed at periodontal biomarkers, proteomics, and peptidomics based on mass spectrometry have become promising methods that provide strong insights into the understanding the inflammation and tissue destruction processes of periodontal diseases (Bostanci & Bao, 2017; Guzman et al., 2014; Kerishnan et al., 2016). Several proteomic studies have demonstrated that S100 proteins in saliva and GCF were at higher level in periodontitis patients compared with individuals without periodontitis (Carneiro et al., 2014; Haigh et al., 2010; Shin et al., 2018; Yaprak et al., 2018). Using nanoflow liquid chromatography tandem mass spectrometry (LC-MS/MS), Guzman et al. (2018) identified azurocidin, lysozyme C, and myosin-9 as baseline biomarkers and α-smooth muscle actin as a candidate biomarker at 13 weeks after treatment. Peptidome is not only the sum of bioactive peptides or degradation products of proteins, but also includes essential elements in plenty of biological processes and is considered a rich reservoir for the discovery of new biomarkers (Amado et al., 2010; Hu et al., 2009; Schrader, 2018; Vitorino et al., 2014). Salivary peptidome profiling analysis based on magnetic beads has been used for the diagnosis of Sjögren's syndrome (Wei et al., 2013) and for discriminating orthodontic patients with periodontitis from those without periodontitis (Zhang et al., 2012). Our previous study (Tang et al., 2019) focused on the peptidome of saliva, serum, and GCF between individuals with periodontitis or not. The results showed that the majority of the peptides from patients with periodontitis was higher in abundance.

Although peptidomics for periodontitis markers was gradually developing, few studies had focused on the peptidome of saliva, GCF, and serum pre- and post-SRP in patients with periodontitis. Hence, this work aims to investigate and characterize the peptidome along with the changes in non-surgical periodontal treatment and simultaneously identify candidate biomarkers for the surveillance of periodontitis.

2 | MATERIALS AND METHODS

2.1 | Ethic approval and study population

The study was performed under the approval by the Ethics Committee of Peking University (Ethics number: IRB00001052-16072) and conducted from March 2017 to September 2017. Written informed consent was signed by all participants before the study commenced.

The inclusion criteria of study population were as follows: (1) over 20 years of age, (2) in good state of general health, and (3) having more than 20 teeth (except the third molars). Exclusion criteria for this study included the following items: periodontal therapy within 12 months, smoking, suffered from systemic diseases that could influence the course of periodontal diseases (e.g. acute cardiovascular events in the 12 months before the start of the study, diabetes, renal failure, rheumatoid arthritis, or liver dysfunction), medications of

topical or systemic antibiotics within 3 months, under orthodontic treatment, oral mucosal inflammatory condition, or serious caries.

2.2 | Clinical evaluation and non-surgical periodontal treatment

All periodontitis subjects received a full-mouth dental examination, and their medical and dental histories were recorded at every appointment for sampling. The clinical periodontal indices, including probing depth (PD), bleeding on probing (BOP) (Lang et al., 1990), bleeding index (BI) (Mazza et al., 1981), and clinical attachment loss (CAL), were recorded by a specialized examiner using a Williams graduated periodontal probe.

A total of 17 patients with the clinical diagnosis of periodontitis were recruited for this study. All patients comply with the definition of generalized periodontitis (Stage I/II) with regard to the extent and severity due to the early onset of periodontal diseases, which diagnosis was in accordance with the clinical criteria stated in the consensus report of the World Workshop in Periodontitis (Caton et al., 2018; Chapple et al., 2018). These patients had at least 30% of teeth with CAL ≥ 1 mm and PD ≥ 4 mm. Non-surgical periodontal therapy, including supragingival ultrasonic instrumentation, subgingival scaling, and root planning using hand instruments (Gracey curettes 5/6, 7/8, 11/12, 13/14; Hu-Friedy) under local anesthesia if needed, was performed by periodontists from Peking University Hospital of Stomatology with quality control inspected by the same clinical instructor. The appointments for the periodontal examination and sampling of every participant were set at baseline (T_0), 7 days after supragingival scaling (T_1), and 8 weeks after the completion of SRP for re-evaluation (T_2). No antibiotics medication during the therapy period.

2.3 | Sample collection

Serum samples were collected at T_0 and T_2, while saliva and GCF samples were collected at all the three visits (T_0, T_1, and T_2). After participants had refrained from oral hygiene measures and food intake for 2 hr in the morning, the sampling procedures were performed. Every participant was instructed to rinse mouth with tap water and rest for 10 min before sample collection. Sample collection steps were as follows:

1. Saliva: Unstimulated whole saliva samples were collected, placed on ice, and then centrifuged at 10,000 *g* for 10 min at 4℃. The supernatant of every sample was divided into 150 μl aliquots and stored at −80℃.
2. GCF: Each participant contributed to one pooled GCF sample from six sampling teeth (11, 31, 16, 26, 36, and 46). After cotton isolation of the sampling site to avoid contamination with saliva, the supragingival plaque was gently removed and the tooth was air dried. Then, a 30-s GCF sample was collected on paper strips (Whatman Lab Sales Ltd.). The strips were gently inserted into the sulcus or pocket, 1–2 mm subgingivally. All strips with GCF samples from each participant were immediately transported into separate coded Eppendorf tubes containing 350 μl of sterile phosphate-buffered saline (PBS) and then centrifuged at 10,000 *g* for 10 min at 4℃ to obtain the supernatants which were stored at −80℃. The GCF collection was carried out before clinical evaluation, and the strips with blood contamination were discarded.
3. Serum: After overnight fasting, 5 ml of venous blood was obtained from each individual. The samples were left at room temperature until the blood clotted, and then the serum samples were collected and stored by means of 100 μl aliquots at −80℃.

2.4 | Weak cation exchange fraction and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

All samples were fractioned using the magnetic beads (MB) kit (Bioyong Technologies Inc.) based on weak cation exchange (WCX) principle. After isolation and purification procedures, the clear supernatant was transferred to labeled tubes for further analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

8 mg/ml α-cyano-4-hydroxycinnamic acid (CHCA) was dissolved in 50% acetonitrile/0.1% trifluoroacetic acid (TFA)/49.9% deionized water to prepare for the matrix solution. Firstly, 1 μl of the extracted peptides solution was added to MALDI-TOF MS target plate of Clin-ToF mass spectrometry system (Bioyong Technologies Inc.) and dried at room temperature. Then, 1 μl of matrix solution was applied to cover the sample and dried again before MS. Before analysis, a three-peptide mixture (monoisotopic molecular weights of 1533.8582, 2465.1989, and 5730.6087 Da; product numbers P2613, A8346, and I6279, respectively; Sigma-Aldrich) was applied to calibrate the MALDI-TOF MS. Positive-ion-200 μm was set as ion source, and 400 shots of laser energy were used. The peptides with molecular weight ranging from 1,000 to 10,000 Da were recorded. Each sample was analyzed three times to get the mean values of the intensities of every peptide peak.

2.5 | Statistical analysis

The BioExplorer (Bioyong Technologies Inc.) software was used to analyze data collected from the MS system. Before analysis, BE was set to subtract baseline, normalize, and align the spectra with signal-to-noise (S/N) ratio >5, a mass shift of no more than 0.1%. The peak area was calculated for quantitative standardization.

The peptide data were analyzed by BE statistical package, while the statistical analysis for clinical parameters was conducted by SPSS v22.0 (IBM). Paired Student's *t* tests and the Wilcoxon signed-rank test were used for evaluation of differences in peptide levels and clinical parameters among the three time points based on the results of normality tests. Cluster analysis and three-dimension scatter

analysis were carried out using STAMP (v2.1.3) (Parks et al., 2014) and Origin 2018 (OriginLab Corp.), respectively. The threshold for statistical significance was defined as $p < .05$.

2.6 | Identification of differentially expressed peptides by nano-liquid chromatography-electrospray ionization-tandem mass spectrometry

The sequences of candidate peptide biomarkers were identified using nano-liquid chromatography-electrospray ionization-tandem mass spectrometry (nano-LC/ESI-MS/MS). After separated by nano-UPLC system EASY-nLC1000 (Thermo Fisher Scientific), the peptides solution was passed through a needle in the ESI source with a high voltage of 3.5 kV. Then, the ions were accelerated into the Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific) for subsequent measurement. To fragment the peptides, higher-energy collision dissociation (HCD) was performed with the analysis time of 120 min. The mass-to-charge ratio (m/z) values of peptides were obtained through 20 fragmented fingerprints after every full scan. An initial screening was performed after inputting the MS/MS raw file into Proteome Discoverer software v1.4 (Thermo Fisher Scientific).

The parameters set are shown as follows: the mass range of the parent ion was 350–6,000 Da and the minimum number of peaks in MS/MS figures was set as 10. The threshold of S/N ratio was 1.5. After initial screening, the mass spectra were searched using Mascot software (v2.3.2) based on dynamic modifications (Oxidation [M]) and static modifications (Carbamidomethyl [C]). Peptide mass tolerance was set as 20 ppm, and fragment mass tolerance was 0.1 Da. The database NCBInr 20120419 (17893860 sequences, 6141683785 residues) with false discovery rate (FDR) ≤1% was applied for analysis.

3 | RESULTS

3.1 | Demographics and clinical parameters

Seventeen Chinese patients of Han ethnicity aged 23 ~ 61 years old were enrolled in this study. As shown in Table 1, all periodontal parameters were significantly different between T_0 and T_2, as well as T_1 and T_2. There were no significant differences between T_0 and T_1.

TABLE 1 Full-mouth periodontal parameters of the subjects ($n = 17$, male: female = 8:9, mean age: 40.12 ± 11.60) from T_0 to T_2

Clinical parameters	T_0	T_1	T_2
BOP (%)	55.59 ± 23.89	50.39 ± 20.92	30.27 ± 13.13*,†
BI	2.34 ± 0.85	2.21 ± 0.69	1.63 ± 0.49*,†
PD (mm)	2.72 ± 0.67	2.66 ± 0.47	2.18 ± 0.27*,†
CAL (mm)	0.31 ± 0.32	0.26 ± 0.19	0.03 ± 0.03*,†

Note: T_0, baseline. T_1, the time point right after supragingival scaling. T_2, 8 weeks after non-surgical treatment. p value: Wilcoxon signed-ranks test.

*Statistical significance compared with T_0.

†Statistical significance compared with T_1.

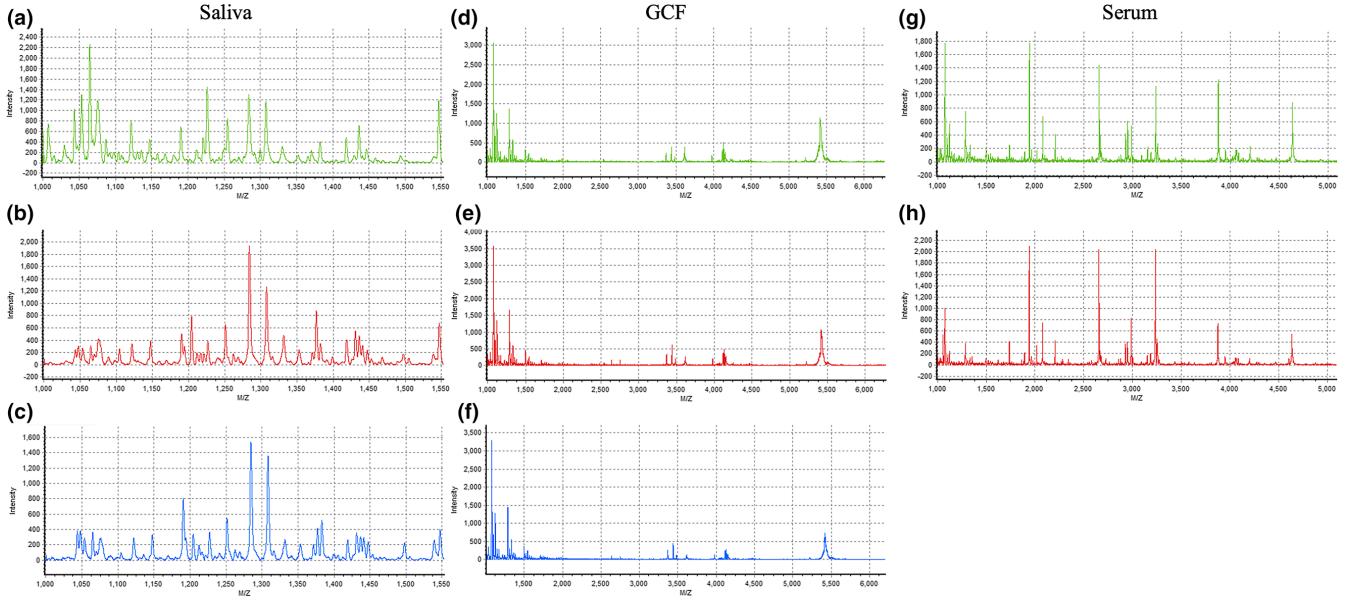

FIGURE 1 Peptide mass fingerprint of characteristic participants. (a, b, c) peptide mass fingerprint of saliva; (d, e, f) peptide mass fingerprint of GCF; (g, h) peptide mass fingerprint of serum (a, d, g: T_0; b, e: T_1; c, f, h: T_2)

3.2 | Change of Peptide profiles during the course of non-surgical periodontal treatment

A total of 136 samples were collected from the 17 participants with stage I / II generalized periodontitis at baseline (T_0), the visit after supragingival scaling (T_1), and the visit after 8 weeks of treatment (T_2). Saliva, GCF, and serum peptides fingerprint peaks were quantified according to the maximum intensity within a particular *m/z* range (Figure 1).

The peak intensities differed significantly for 9 peptides in saliva (experimental *m/z* values: 1,065.6, 1,075.9, 1,122, 1,147.1, 1,284.3, 1,292.8, 1,418.7, 1,494.7, and 1,547 Da; Figure 2). For the peptidome of GCF, 9 peptide peaks were down-regulated (experimental *m/z* values: 1,032.6, 1,285.6, 1,308, 1,506, 3,366.5, 3,437.6, 5,397.8, 5,410.3, and 5,419.1 Da). Four peptides in serum were decreased between baseline and 8 weeks after SRP (experimental *m/z* values: 1,286, 2,946.3, 3,874.9, and 4,635.2 Da). All of these differentially expressed peptides were down-regulated (Figure 2).

3.3 | Use of peptidome in distinguishing different periodontal treatment status

Cluster analysis was carried out according to the intensities of significantly different peptides of every periodontitis participant in response to treatment (Figure 3). First, the cluster analysis of salivary peptides between T_1 and T_2, and T_0 and T_2 showed more obvious separation than did the comparison between T_0 and T_1. Second, differentially expressed peptide peaks from saliva exhibited better cluster capacity of the samples, whereas peptides from GCF and serum could not distinguish the subjects.

Using all of these peptides, we established three-dimensional scatter plots and found that the plot formed by 1,147.1, 1,284.3, and 1,418.7 Da peptides from saliva exhibited a separation tendency between T_0 and T_2 (Figure 4). Their three-dimensional *m/z* plots are shown in Figure 5.

3.4 | Identification of candidate peptide biomarkers

With nano-LC-ESI-MS/MS, seven of the differentially expressed peptides were successfully identified (Table 2). The peptides with experimental *m/z* values of 1,122.0 and 1,494.7 Da from saliva were predicted to be segments of immunoglobulin κ variable 4-1, while those with values of 1,286 Da from serum and 1,285.6 Da from GCF were identified as immunoglobulin heavy constant γ2 peptides. In addition, three peptides (experimental *m/z* values: 1,075.9 and 1,147.1 Da from saliva, 3,874.9 Da from serum) were derived from α-1-antitrypsin and haptoglobin, respectively.

4 | DISCUSSION

Periodontitis is an advanced destructive disease of the periodontium that is highly prevalent worldwide and is one of the two common oral

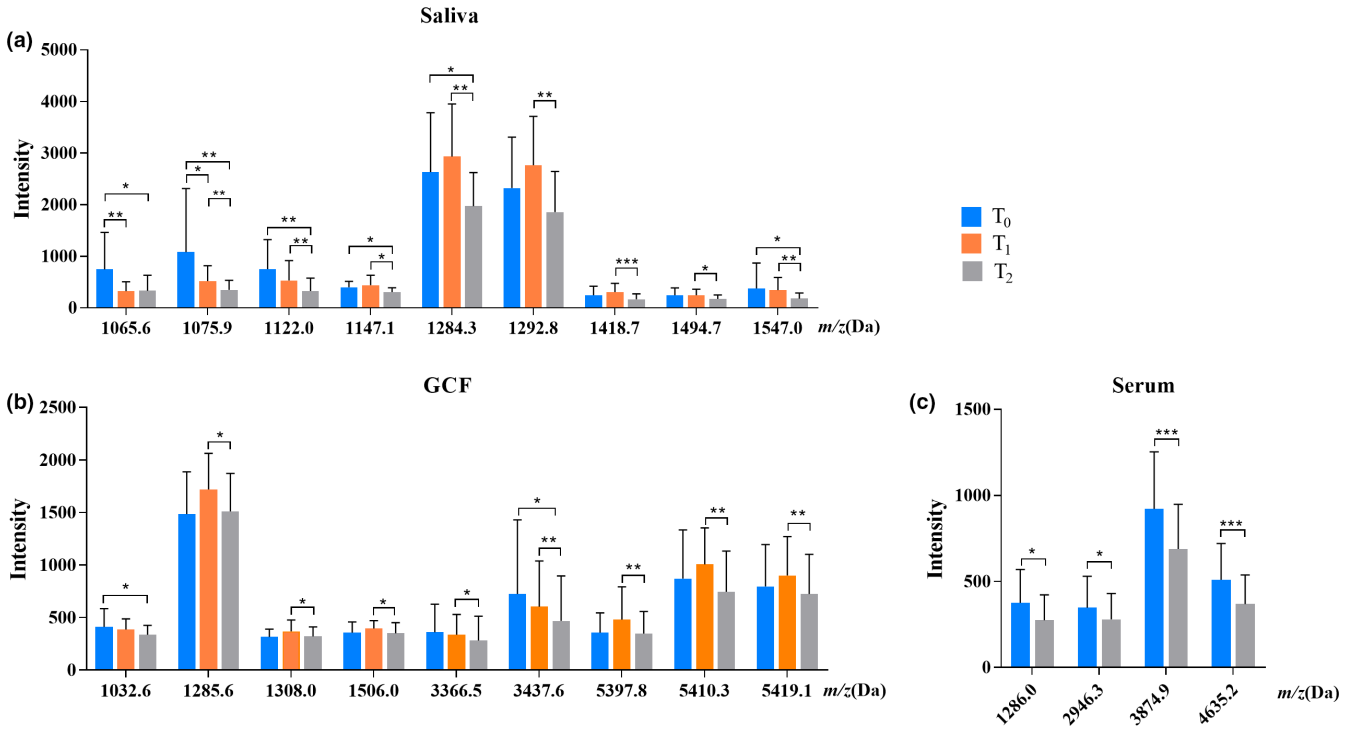

FIGURE 2 Histogram of intensities of significantly different peptides in saliva (a), GCF (b), and serum (c) over the experimental period. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$

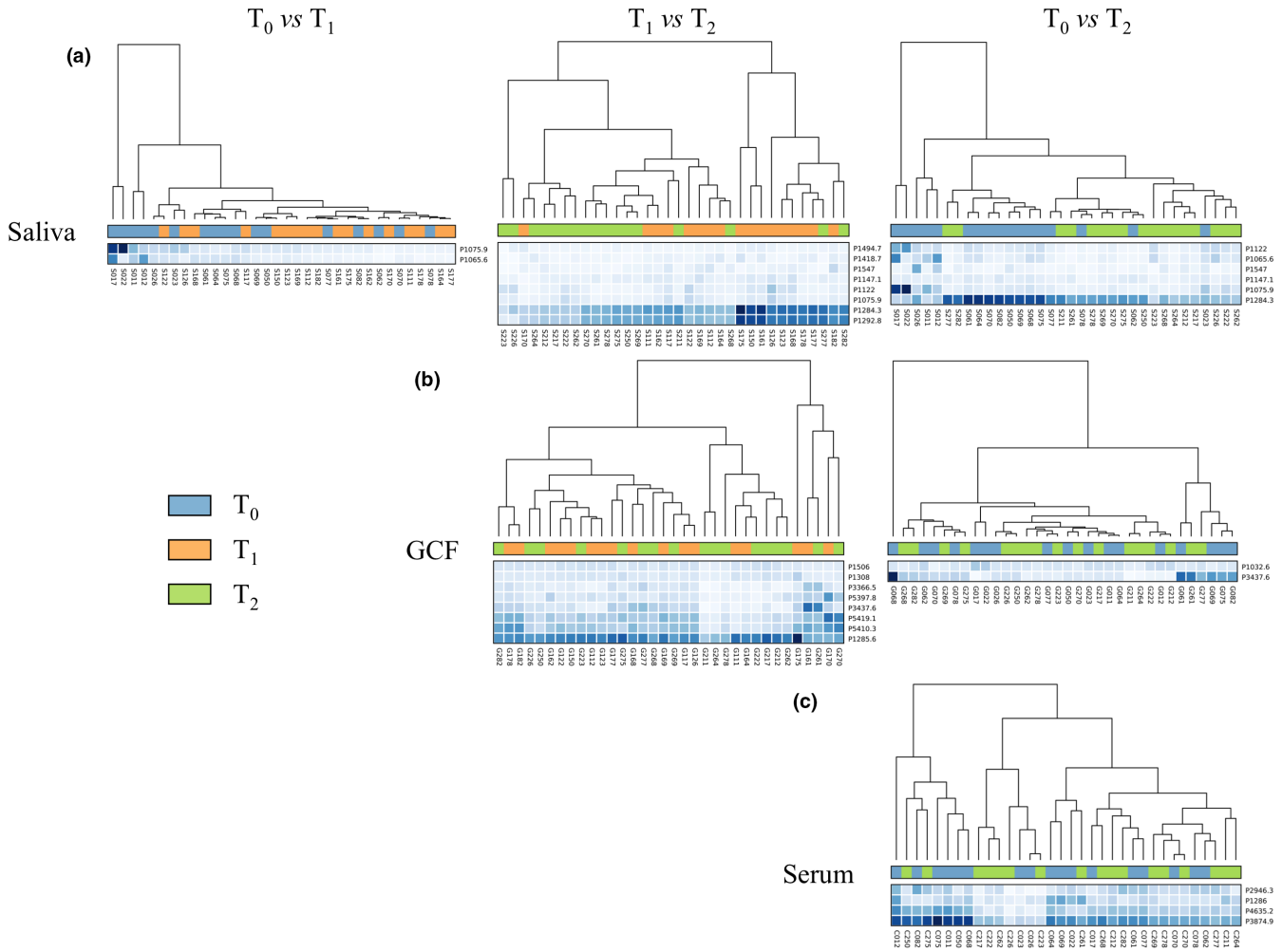

FIGURE 3 Cluster analyses of the significantly different peptides in saliva (a), GCF (b), and serum (c) with regard to the distribution of the subjects among the three time points

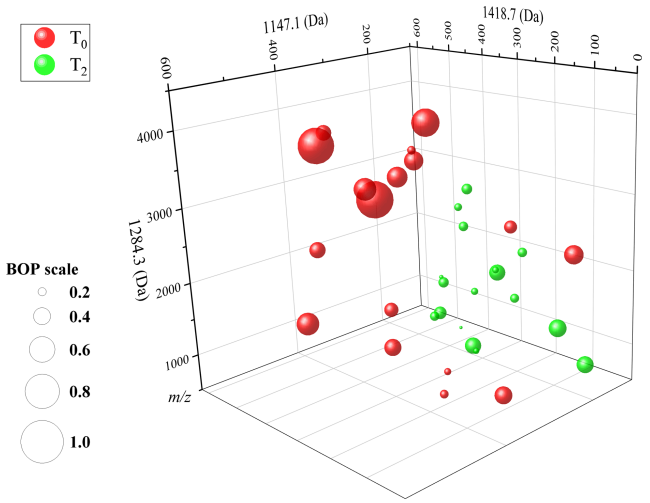

FIGURE 4 Three-dimensional scatter plots of the periodontitis patients at T_0 and T_2, adopting peptides with *m/z* of 1,147.1, 1,284.3, and 1,418.7 Da. Red represents T_0; green, T_2. The size of the spheres is determined by the BOP value of the participant, which is demonstrated by the scale range

diseases which contributes to the chronic disease burden globally (Kinane et al., 2017; Petersen & Ogawa, 2012). At present, the diagnosis and monitoring of periodontitis are mainly based on medical history, clinical examination, and radiographic examination (Caton et al., 2018; Chapple et al., 2018). There are also many studies focusing on the use of immunoassays to explore potential disease markers in saliva and GCF (Barros et al., 2016; Lorenzo-Pouso et al., 2018; Pradeep et al., 2011). However, the aforementioned methods require training and are time-consuming in large-scale screening, and insufficient for complex immune-inflammatory diseases, such as periodontitis. As the amount and profile of peptides are modulated by physiological and pathological states, proteomics and peptidomics can be used to detect specific markers to obtain a comprehensive understanding of cellular pathways modulated by diseases (Menschaert et al., 2010; Michael Schrader et al., 2014; Vitorino et al., 2014). MALDI-TOF MS based on magnetic beads has the characteristics of easy sample preparation, low sample consumption, high sensitivity, and a high degree of automation in high-throughput analyses of a complex biological sample, which means great clinical application value (Cho et al., 2015; Hajduk et al., 2016). This study

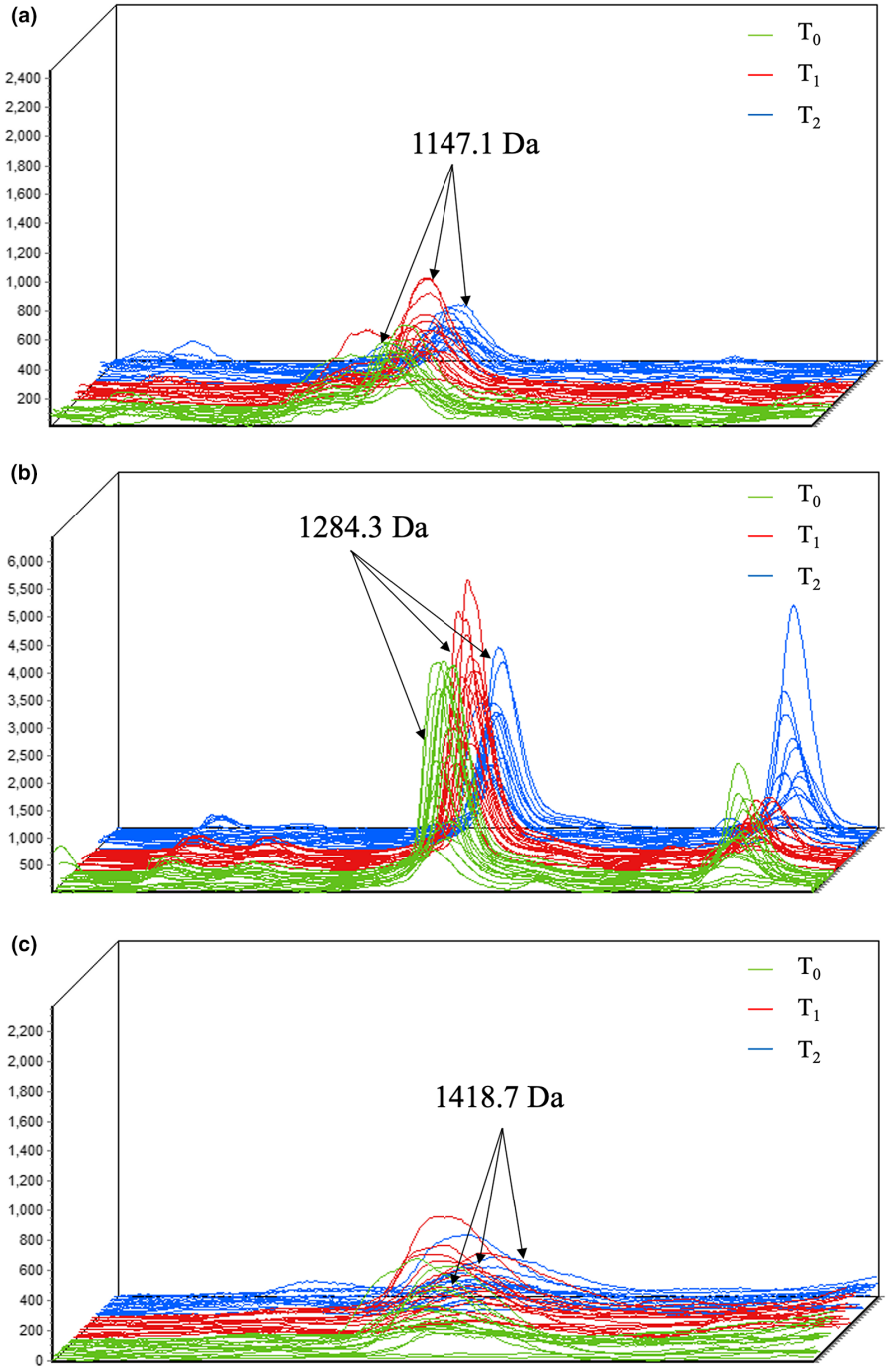

FIGURE 5 Three-dimensional mass fingerprints indicating intensities alteration of peptides at 1,147.1, 1,284.3, and 1,418.7 Da in saliva among three time points. Green: T_0; red: T_1; blue: T_2

used MALDI-TOF MS to explore the potential peptide biomarkers before and after the treatment of generalized periodontitis (stage I/II) to seek for ways to monitor the changes of periodontal status by detection of body fluids.

In one previous cross-sectional research conducted by our research group (Tang et al., 2019), in total 7 salivary peptides and 13 serum peptides were detected to be higher in the CP group compared with the healthy subjects, with 2 of these salivary peptides (*m/z* values: 1,122.0 and 1,147.1 Da, identified as immunoglobulin κ variable 4-1 and haptoglobin, respectively) and 3 of these serum peptides (*m/z* values: 2,946.3, 3,874.9 and 4,635.2 Da, with the peptide 3,874.9 Da identified as a segment of haptoglobin) also detected in the present study, which average intensities showed a decreasing trend after periodontal treatment as a reflection of the improvement in periodontal status. As we were performing self-control comparisons between pretreatment and post-treatment which might undergone less influences by individual specificity, the present results of our present study could yet demonstrate the changes brought by the periodontal treatment, though a re-examination for the healthy subjects at the time point when the disease group finished non-surgical periodontal treatment for 8 weeks was not performed in consideration of ethic requirements that minimize potential impact on the participants.

Seventeen participants with generalized periodontitis (stage I/II) were enrolled in this study and their saliva, GCF, and serum were

TABLE 2 Identification of peptide peaks as candidate biomarkers

Sample	Experimental *m/z* value	Theoretical *m/z* value	Protein name	Peptide sequence
Saliva	1,075.9	1,076.61726	α-1-antitrypsin	LSSWVLLMK
	1,122.0	1,122.63061	Immunoglobulin κ variable 4-1	LLIYWASTR
	1,147.1	1,146.54258	Haptoglobin	HYEGSTVPEK
	1,494.7	1,495.6787	Immunoglobulin κ variable 4-1	YYCFQGNQFLR
GCF	1,285.6	1,286.67393	Immunoglobulin heavy constant γ2	EPQVYTLPPSR
Serum	1,286.0	1,286.67393	Immunoglobulin heavy constant γ2	EPQVYTLPPSR
	3,874.9	3,874.67011	Haptoglobin	YQEDTCYGDAGSAFAVHDLEEDTWYATGILSFDK

Abbreviation: *m/z*, mass-to-charge ratio.

collected, and differentially expressed peptides before and after non-surgical periodontal treatment were analyzed. Nine salivary peptide peaks, nine GCF peptide peaks, and four serum peptide peaks were down-regulated. More specifically, six salivary peptides, two peptides from GCF, and four peptides from serum were significantly decreased in the comparison between T_0 (baseline) and T_2 (8 weeks after treatment). A comparison of T_1 with T_0 revealed that only 2 peptide peaks (*m/z* values: 1,065.6 and 1,075.9 Da) in saliva were significantly down-regulated. No differential peptide peaks were found in the GCF sample between T_0 and T_1. (Ongoz Dede et al., 2017) collected saliva and GCF samples from 27 subjects with periodontitis and measured IL-10, IL-32, and TNF-α levels by ELISA. The results showed that the levels of TNF-α and IL-32 in saliva and GCF were decreased in patients with periodontitis before and after treatment ($p < .05$), while the levels of IL-10 were significantly increased. (Trindade et al., 2015) compared the salivary peptides of healthy controls with those of periodontitis patients by nano-HPLC-MALDI-TOF/TOF MS. These authors found eight acidic PRP and P-B peptides that were correlated with periodontitis and exhibited potential predicted antimicrobial activity. (Salazar et al., 2013) applied LC-MS/MS to investigate the proteome profiles of the whole saliva from 20 periodontally diseased participants and 20 periodontally healthy controls. As for protein abundance between controls and patients, 20 proteins were found a 1.5-fold difference, most of which were higher in patients. In addition, the functional analysis of these proteins indicated the connection between the status of periodontal diseases and inflammatory processes. De Souza et al., (2017) studied serum C-reactive protein before and after non-surgical periodontal treatments. The results showed that 60 days after basic treatment, serum CRP levels were significantly decreased. In this study, a comparison of the peptidome between T_0 and T_1 revealed only two peptides from saliva samples were significantly expressed, namely 1,065.6 and 1,075.9 Da. More differentiated peptides occurred 8 weeks after non-surgical periodontal treatment. When the samples were collected at T_1, patients with periodontitis had finished supragingival scaling, but marked amounts of calculus and microbial biofilms remained under the gingival sulcus for subsequent removal by subgingival scaling. Considering the pathological aspects of periodontal diseases, periodontitis was initiated and sustained by microorganisms of dental plaque (Kinane et al., 2017; Pihlstrom et al., 2005). Mechanical removal of dental plaque and calculus, both supragingival and, in particular, subgingival, was the key to the successful treatment of individuals with periodontal disease (Drisko, 2001; Kinane et al., 2017; Krishna & De Stefano, 2016). Therefore, it was speculated that after the complete removal of subgingival plaque and calculus, changes in the oral microenvironment could be reflected in the corresponding peptides of saliva. This study found none significantly different peptides in the GCF samples between T_1 and T_0. This discrepancy is possible because the treatment of periodontal inflammation requires the destruction and removal of the subgingival dental plaque (Drisko, 2001; Kinane et al., 2017; Krishna & De Stefano, 2016), and peptidome changes in the periodontal pocket microenvironment may not be detected in patients who have not yet undergone thorough subgingival scaling.

The cluster analysis demonstrated that differentially expressed peptides in saliva have a better classification in patients with periodontitis in response to treatment. As shown in Figure 3, with the clinical improvement of individuals, peptides at different time points exhibited a certain clustering tendency. Unlike the salivary peptide cluster analysis between T_0 and T_1, the separation tendency of individuals was obvious when T_2 was compared with T_0 or T_1. Therefore, the cluster analysis results of saliva samples indicated that complete non-surgical periodontal treatment caused significant changes in the local peptidome. However, differentially expressed peptides in GCF and serum samples before and after subgingival scaling or complete mechanical therapy showed poor clustering trends. Thus, the changes in GCF and serum peptidome profiles may not be enough to effectively reflect the impact of treatment. The cluster analysis was used to preliminarily distinguish the differentially expressed peptides from different periodontal disease statuses and uncover the fine cluster capacity of salivary peptides. Moreover, a scatter plot established by 3 salivary peptides (*m/z* values: 1,147.1, 1,284.3 and 1,418.7 Da) showed that the individuals were more concentrated after non-surgical periodontal treatment. Such tendency was not found in scatters plots using other peptides in GCF or serum. We presumed that these results might provide initial evidence for these salivary peptides acting as potential candidate biomarkers of generalized periodontitis (stage I / II), especially associated with treatment outcomes.

With nano-LC/ESI-MS/MS, several peptides were successfully identified (Table 2). The 1,122 Da and 1,494.7 Da peptides in saliva were identified as segments of immunoglobulin κ variable region 4-1, the V segment of the immunoglobulin light chain variable region, which is involved in the recognition process of antigen (Lefranc, 2014). This protein showed a downward trend after periodontal treatment, presumably related to the reduced activity of the host–pathogen immune response. Two peptides in GCF and serum samples, namely 1,286 Da and 1,285.6 Da, respectively, were detected as segments of the constant region of immunoglobulin heavy chain γ2. Immunoglobulins are heterodimeric proteins consisting of two heavy chains and two light chains, all of which are functionally divided into variable domains and constant domains. The former domains bind antigens, while the latter domains specify effector functions, such as the activation of complement (Schroeder & Cavacini, 2010). In this study, the expression of immunoglobulin peptide fragments in saliva, GCF, and serum was down-regulated after periodontal treatment, suggesting changes in the host immune response.

The 1,075.9 Da peptide in saliva was identified as a segment of α-1-antitrypsin (AAT), which is highly expressed in periodontal disease before treatment. Protease inhibitors are anti-inflammatory reactants. Serum-derived AAT (a protease inhibitor) is enhanced by inflammatory cytokines and endotoxin regulation, and AAT is also considered an inflammation-related molecule (Janciauskiene et al., 2011). A previous study found that an AAT deficiency might be related to increased susceptibility to periodontitis (Peterson & Marsh, 1979). Using MALDI-TOF MS, Preiano et al. (2018) compared the GCF peptides from 10 gingivitis patients and 10 healthy controls and found that the intensities of peptide peak with an *m/z* value of 4,136 Da was elevated in patients with gingivitis. The 4,136 Da peptide was identified as a C-terminal fragment derived from AAT, which inhibits the degradation of periodontium by preventing neutrophil elastase in GCF. Our study did not find changes in the corresponding peptides in GCF. However, previous studies on the levels of AAT in saliva and serum demonstrated no significant association with periodontitis (Pederson et al., 1995; Sandholm et al., 1981; Scott et al., 2002; Wallin-Bengtsson et al., 2011). Further exploration and validation are still needed for AAT.

Haptoglobin is a human plasma protein which binds firmly to hemoglobin during hemolysis (Andersen et al., 2017). It is also an acute-phase protein with a broad anti-inflammatory effect, mostly synthesized by the liver. Due to the capacity to bind free hemoglobin and promote its clearance by macrophages, haptoglobin can act as a bacteriostatic agent and an antioxidant indirectly (Theilgaard-Monch et al., 2006). Besides, Langerhans cells in the epithelium can also synthesize haptoglobin, which increases its expression in local or systemic inflammation and injury (Li et al., 2005; Theilgaard-Monch et al., 2006). Haptoglobin can promote body repair during local or systemic infection by mediating a series of anti-inflammatory processes. This study found that the 1,147.1 Da peptide in saliva and the 3,874. 9 Da peptide in serum were derived from haptoglobin. Ebersole (Ebersole et al., 1997) found higher levels of haptoglobin in periodontitis patients compared with healthy controls, which could be the result of infection burden or inflammation in patients with periodontitis. However, another animal experiment from these authors (Ebersole et al., 1999) indicated that serum haptoglobin was not affected during the experimental periodontitis phases. Haigh (Haigh et al., 2010) compared the composition of saliva before and after the treatment of periodontitis and found higher haptoglobin levels before treatment. A longitudinal study in dogs, attempting to find candidate biomarkers of periodontitis progression, also demonstrated the upregulation of haptoglobin in GCF and confirmed the results by ELISA measurements (Davis et al., 2016). Thus, haptoglobin levels may reflect increased anti-inflammatory and periodontal tissue repair processes. In this study, the final clinical evaluation (re-evaluation) and sample collection were performed 8 weeks after SRP in periodontitis participants because most of the healing occurs at this same time (Badersten et al., 1981). The mean PD, BI score, and CAL of the periodontitis patients were lower after periodontal treatment, which was commensurate with that of the intensities of significantly different peptides in saliva, serum, and GCF.

In the present study, different biomarkers were detected in the three types of body fluids, particularly for saliva and GCF, which also exhibited different peptide peaks in comparison between pretreatment and post-treatment time points (Figures 1 and 2, Table 2). This finding could be interpreted by the different source and secretion way of saliva and GCF, with the former serving as a reflection of the whole mouth while the latter associated more with specific sites. Nevertheless, our results indicated that certain peptides in saliva could also vary between different timepoints before and after non-surgical periodontal treatment, demonstrating the potentiality for using saliva samples in assessment of prognosis and surveillance of progression of periodontal diseases. From a practical perspective, saliva could be collected without specialized knowledge and provide a quick assessment of the oral environment, whereas GCF collection was more technically challenging with much smaller amount and involved 168 possible sampling sites at most, making it difficult to achieve rapid full-mouth sampling and evaluation (Guzman et al., 2014). Besides, for some special population, it might not be so feasible to collect GCF as expected, under which circumstances saliva could be an appropriate substitution. As serum sampling was also faced with the problem of invasiveness and poor compliance, saliva still had its own advantages and application values in both clinical settings and communities for monitoring the status of periodontal diseases.

Some limitations of this investigation need to be realized. First, this study only focused on mild-to-moderate generalized periodontitis (stage I/II), which findings need further verification to be extrapolated to localized periodontitis. This selection of study subjects was based on our thoughts to reduce the potential impact on salivary peptidome caused by the severity of periodontitis, as well as to ensure the sensitivity of these differentially expressed peptides as candidate biomarkers for non-surgical periodontal treatment. Second, the lack of plaque indices was also an important limitation that hindered us from comparing the periodontal status more comprehensively,

which should be taken into account in further in-depth studies. Also, though we were comparing the differences in peptide levels and clinical parameters between different time points individual specifically in the present research, it would be better if the participant's age could be employed as a grouping factor in future studies with a larger sample size. Third, the identities and underlying regulatory processes of some peptides remained unclear. Although peptides acted as "information carriers" indicating the condition of part of or an entire organism since they were generated after the metabolism or other obscure proteolytic degradation of large proteins by a variety of peptidases (Magalhaes et al., 2018; Schrader, 2018; Schrader & Selle, 2006), these molecules were the comparatively complex and challenging biomolecules to be identified. With the ever-developing peptidomic technologies and enriched databases, it would be able to further unravel the disease surveillance capabilities of candidate biomarkers some day in the future. Fourth, though in the present study we were focusing on saliva most and a pilot study had confirmed the amount of GCF varied very little for each participant among different time points in the course of non-surgical periodontal treatment, the GCF samples we used were collected from six index teeth with the concentrations not precisely quantified. Further studies using GCF collected from those teeth with highest severity of periodontitis with more precise quantification of the concentration could exhibit more accurate peptidome characteristics of GCF and its relationship with periodontal status.

5 | CONCLUSIONS

The results in this study indicated that certain peptides in saliva, serum, and GCF were down-regulated after non-surgical periodontal treatment of generalized periodontitis (stage I/II), demonstrating the application prospects of saliva in monitoring and surveillance of periodontal diseases in both clinical settings and communities, which also lay a foundation for future studies on the relationship between salivary peptidome and oral diseases with the development and improvement of peptidomic techniques and databases.

CONFLICT OF INTEREST

None to declare.

AUTHOR CONTRIBUTIONS

Chao Yuan: Conceptualization; Investigation; Project administration; Supervision; Writing-review & editing. **Zhangke Ma:** Data curation; Investigation; Visualization; Writing-original draft. **Peiyuan Tong:** Data curation; Investigation; Methodology. **Shunlan Yu:** Methodology; Project administration; Supervision. **Yi Li:** Conceptualization; Investigation; Project administration. **Jennifer Elizabeth Gallagher:** Conceptualization; Funding acquisition; Project administration. **Xiangyu Sun:** Conceptualization; Funding acquisition; Project administration; Supervision; Writing-review & editing. **Shuguo Zheng:** Conceptualization; Funding acquisition; Project administration; Supervision.

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