Magnetic Bead-based Salivary Peptidome Profiling for Accelerated Osteogenic Orthodontic Treatments

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Objective: *To identify a panel of differentially expressed candidate biomarkers for patients undergoing accelerated osteogenic orthodontics (AOO).*

Methods: *This study included 36 saliva samples taken from six Class III surgical patients at six time points: the date before the corticotomy procedure (T1) and at 1 week, 2 weeks, 1 month, 2 months and 6 months after the procedure (T2, T3, T4, T5 and T6, respectively). After the maxillary dental arch was aligned and levelled, AOO procedures were performed in the maxillary alveolar bone. Saliva samples were used to create peptide mass fingerprints using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) combined with magnetic beads.*

Results: *When the six groups were compared, 182 peaks were significantly different ($P < 0.01$), and the biomarker profiles changed over time. After corticotomy, the mass peaks predicted to be the Apolipoprotein A-I precursor (APOA1) increased sharply in T2 then decreased. The peptides predicted to be complement component 3 decreased in T2, then gradually increased and declined in T6. The peptides predicted to be the vitamin D-binding protein precursor increased in T2, then fell to the preoperative level. The mass peaks of the peptides predicted to be Isoform 1 of the fibrinogen alpha chain precursor (FGA) first decreased, then increased with time.*

Conclusion: *The salivary protein profiles changed with accelerated tooth movement induced by AOO. This method provides a tool for investigating corticotomy-induced accelerated tooth movement in humans, and explored the critical factors responsible for the regional acceleratory phenomenon.*

Key words: *peptidome, saliva; MALDI-TOF MS, corticotomy, accelerated osteogenic orthodontics*

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Young adult patients who have a skeletal facial deformity such as a skeletal Class III malocclusion are often treated with a combination of surgical and orthodontic therapy. In general, presurgical orthodontic treatment for these patients can take up to two years[1] and postoperative orthodontic treatment typically lasts six months to a year[2]. Overall treatment time for these patients is around three years, sometimes even longer.

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An important goal is therefore to shorten treatment time and still meet the facial aesthetic requirements of these patients. A corticotomy that can decrease the duration of orthodontic treatment may be one solution that will meet the needs of these young adult patients. Wilcko et al[3] demonstrated accelerated osteogenic orthodontics (AOO), and this technique included selective alveolar decortication, alveolar augmentation and orthodontic treatment.

Many studies have reported that the AOO procedures could accelerate tooth movement rate and shorten the duration of orthodontic treatment[3,4]. Wilcko et al reported that accelerated tooth movement may be due to the regional acceleratory phenomenon (RAP), in which local osteoclasts are activated and reduce the surrounding bone density[3]. This phenomenon was observed in SD rats undergoing corticotomy-assisted tooth movement by micro CT[5]. Some studies found that corticotomy could induce a localised increase in turnover of alveolar spongiosa which provides additional evidence for RAP[6,7]. A recent study confirmed that the basic mechanism underlying corticotomy is the simultaneous activation of osteoblasts and osteoclasts after mechanical injury[8]. However, the main cause of RAP remains unclear, partly because almost all previous studies on AOO mechanism are animal-based[5,6,8]. And invasive study methods for animal cannot be used for humans. Thus, in this study we used a new and non-invasive tool to investigate the biomarkers responsible for AOO in humans.

Previous studies have identified biomarkers in saliva by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) using the magnetic bead-based peptidome analysis method[9]. For the current study, we also chose MALDI-TOF MS since it is a relatively convenient and highly sensitive method for generating accurate salivary protein profiles. We compared differences in salivary protein mass peaks from different AOO treatment durations to identify a panel of differentially expressed specific candidate biomarkers responsible for RAP.

Materials and methods

Ethics statement

The University Ethics Committee and Competent Authority approved the study protocol and also supervised the process. All patients were provided with information about the proposed treatment and informed consent forms. The study was entered into the Clinical Trial Registry under registration number ChiCTR-ONRC-13004129.

Subjects

In total, 36 saliva samples were taken from six Class III patients (two males and four females) at the time of maxillary corticotomy and five time points thereafter preceding orthognathic surgical correction of the Class III skeletal malocclusion. The screening criteria for all patients were: 18 to 30 years old (mean 20.0 ± 1.5); Class III anterior, canine and molar relationships;

ANB < -4 degrees (mean -4.17 ± 2.32 in cephalometric measurements) or Wits < -6 mm; bilateral extraction of maxillary first premolars and non-extraction in the mandibular arches; mild crowding in the maxillary arch (≤ 3 mm, mean 2.6 ± 1.2). Exclusion criteria were: patients with active periodontal disease, diseases of the oral mucosa, supernumerary teeth or craniofacial syndromes.

Treatment progress

After the extraction of maxillary first premolars, patients were bonded with pre-adjusted edgewise 0.022-inch fixed appliances. The 0.019 inch × 0.025 inch stainless steel archwire was ligated into the maxillary brackets after aligning and levelling the maxillary arch averaging 12 weeks. Corticotomies were performed only on the buccal side of the alveolar bone with a piezo ultrasonic surgery unit (Piezotome, SATELEC, France). During the first part of the procedure, a full thickness mucoperiosteum flap was reflected. Next, parallel vertical incisions were made in the interradicular spaces from the mesial area of the right second premolar to the mesial area of the left second premolar. These incisions cut through the entire thickness of the cortical plate, and no horizontal corticotomy cuts were made. Bone grafting material (Cerasorb, 50-500 µm, 0.5 g, curasan AG, Germany) was applied to cover the decortication areas. The flaps were carefully repositioned and sutured (Fig 1). After 2 weeks, the patients were examined for their first orthodontic adjustment after the procedure, and further adjustments occurred approximately every 4 weeks.

Saliva collection and processing

A standardised protocol was used for the collection, storage, and processing of all samples. We collected saliva at six time points:

T1: the day after the upper teeth were aligned and levelled and before corticotomy;

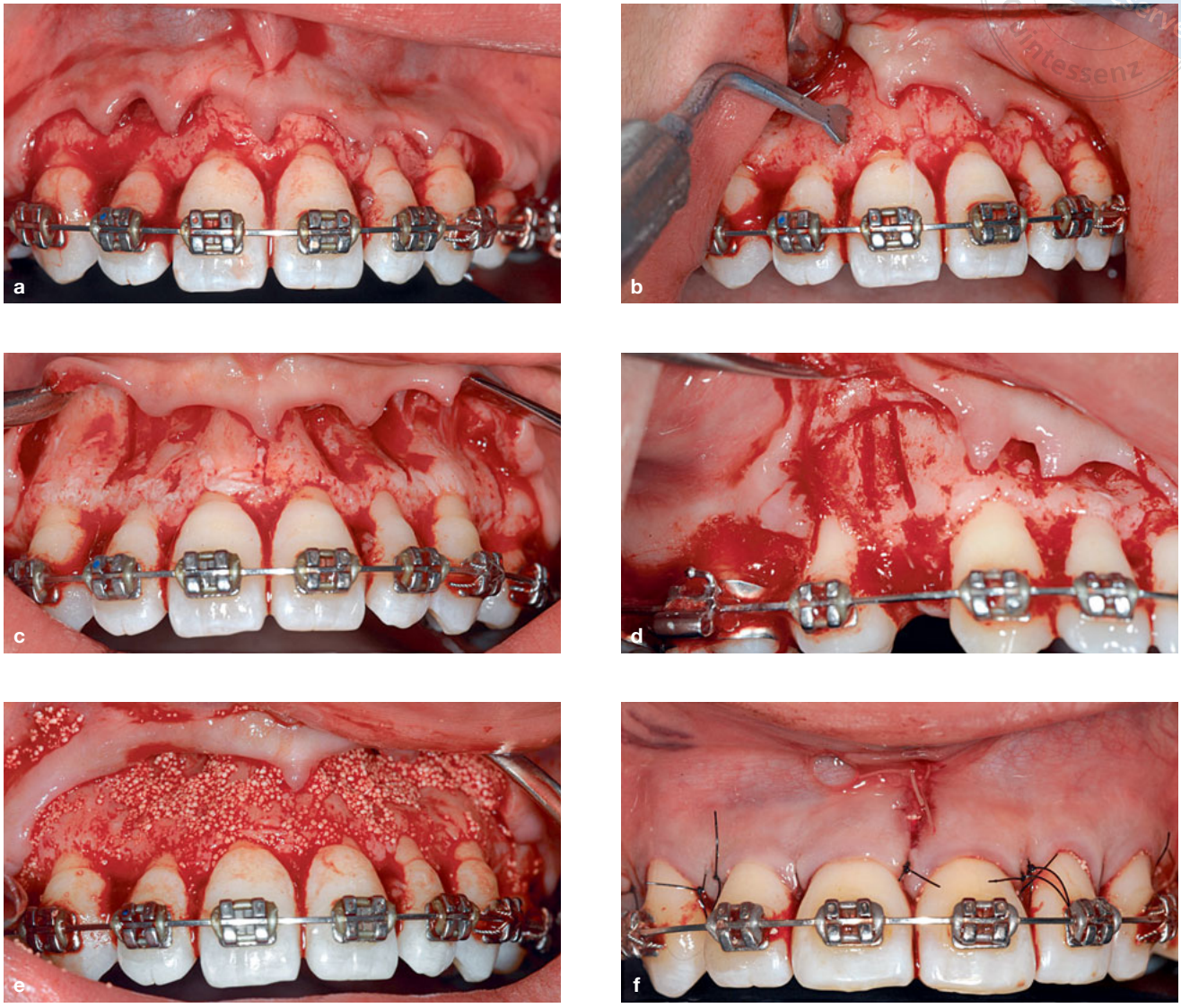

Fig 1 **a)** Reflection of full-thickness flap; **b)** piezosurgery performed on maxillary anterior alveolar bone; **c and d)** interradicular alveolar corticotomy grooves; **e)** bone graft material filled the corticotomy area; **f)** flap was sutured.

T2: 1 week after the procedure;
T3: 2 weeks after the procedure and the date of the first orthodontic adjustment;
T4: 1 month after the procedure and the date of the second orthodontic adjustment;
T5: 2 months after corticotomy and the date of the third orthodontic adjustment;
T6: 6 months after corticotomy.

Unstimulated whole saliva samples were collected between 8 am and 9 am. The subjects were required to put the tip of their tongue against the sublingual caruncle and saliva was collected in a paper cup for the first 3 min. We then used a 50 ml centrifuge tube to collect saliva until 5 ml had been collected. The samples, once collected, were centrifuged at 10,000 g for 10 min at 4°C to remove insoluble materials, debris and cells. Supernatants were immediately allocated into smaller volumes and stored at -80°C.

WCX fractionation and MALDI-TOF MS analysis

All samples were fractionated using weak cation exchange magnetic beads (MB-WCX) via standard protocol according to the manufacturers' instructions. Samples were purified and isolated through three steps: binding, washing, and elution. To begin, 10 µl beads, 10 µl MB-WCX binding solution (BS) and 5 µl samples were added to a tube, mixed carefully and then incubated for 5 min. The tube was placed on a magnetic bead sepa-

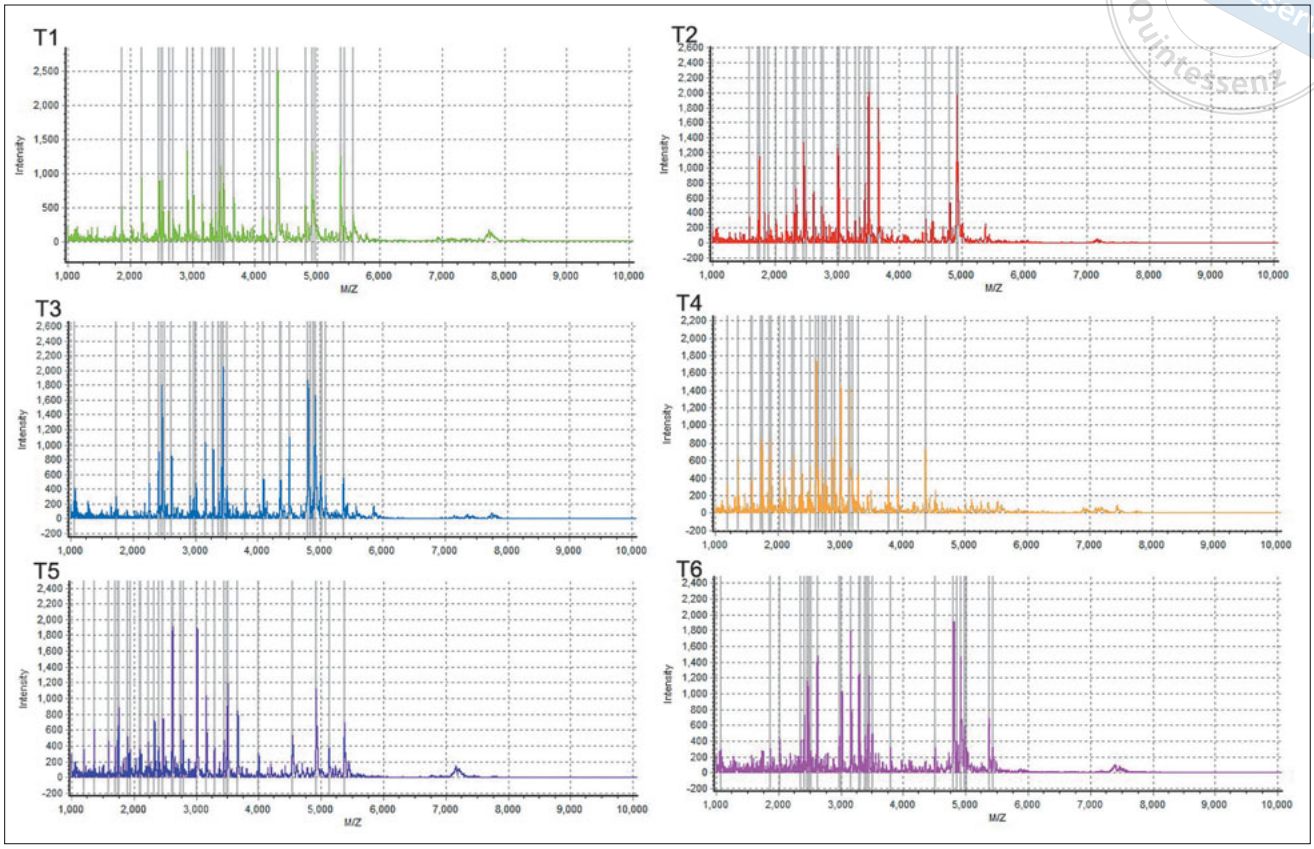

Fig 2 Complete mass spectra in the range of 1000–10000 Da, showing the peptide fingerprints of a saliva sample from a single patient at six time points: T1 (green curve); T2 (red curve); T3 (blue curve); T4 (orange curve); T5 (purple curve); T6 (fuchsia curve). M/z: mass-to-charge ratio.

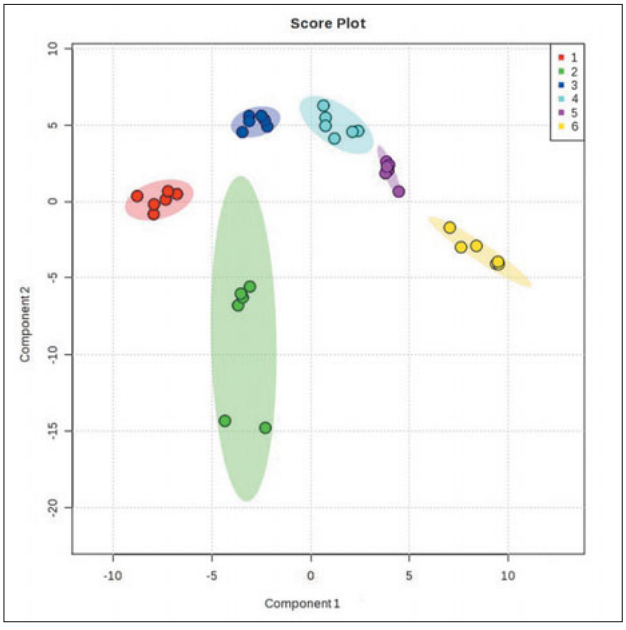

Fig 3 The score plot of the OPLS-DA model among the six groups. T1 (red points); T2 (green points); T3 (blue points); T4 (mint points); T5 (fuchsia points); T6 (yellow points).

ration device (Bruker Daltonics, Billerica, MA, USA) and the beads were collected at the tube wall for 1 min. Next, the supernatant was removed and 100 µl of magnetic bead washing solution (WS) was added and mixed thoroughly. After washing three times, the supernatant was removed, another 10 µl of magnetic bead eluting solution (ES) was added, and the beads were collected at the tube wall for 2 min. After eluting and beating, the magnetic beads were separated from the protein, and the eluted peptide samples were transferred to a 0.2 ml clean sample tube for further MS analysis. Finally, the prepared sample was analysed by MALDI-TOF MS. A range of 1,000 to 10,000 Da peptide molecular weight was collected, and 400 shots of laser energy were used. Peptide mass fingerprints were obtained by collecting 50 single MS signal scans.

Statistical analysis

Analysis of variance (ANOVA)

Data were analysed using the BioExplorer statistical package (BioyongTech, Beijing, China) and Metabo-

Table 1 The peak intensity of peptides that predicted to proteins.

Mean m/z value	*P*-value	Predicted protein	Intensity T1	Intensity T2	Intensity T3	Intensity T4	Intensity T5	Intensity T6
3052.02	< 0.001	Apolipoprotein A-I precursor	122.8 ± 28.0	287.8 ± 72.4	63.3 ± 10.7	52.2 ± 10.2	69.1 ± 23.5	54 ± 22.3
2775.33	< 0.001	Apolipoprotein A-I precursor	292.8 ± 53.0	579.3 ± 138.4	179.5 ± 38.4	163.8 ± 21.9	252.3 ± 26.4	155.6 ± 22.8
1864.17	< 0.001	Complement component 3	232.6 ± 30.6	203.0 ± 41.0	274.8 ± 40.1	381.2 ± 40.9	752.2 ± 76.5	418.1 ± 61.3
2027.72	< 0.001	Complement component 3	344.1 ± 75.1	154.8 ± 44.1	251.8 ± 38.7	233.4 ± 28.2	434.9 ± 63.2	280.0 ± 35.9
2690.66	< 0.001	Vitamin D-binding protein precursor	61.4 ± 6.9	520.5 ± 126.0	71.2 ± 16.7	63.8 ± 8.3	80.0 ± 45.0	64.0 ± 16.1
1473.44	< 0.001	Isoform 1 of Fibrinogen alpha chain precursor	272.7 ± 222.3	59.6 ± 39.2	96.8 ± 46.6	132.1 ± 112.6	120.2 ± 60.8	186.1 ± 76.7
1692.92	< 0.001	Isoform 1 of Fibrinogen or	297.1 ± 176.0	95.4 ± 62.0	154.5 ± 42.9	185.7 ± 124.2	420.2 ± 245.7	523.8 ± 276.6

Analyst 3.0 (http://www.metaboanalyst.ca). ANOVA was used to identify differences in protein concentration among the six groups of saliva samples, and *post-hoc* tests were used for multiple comparisons between groups. Next, the amino acid sequences of significantly different peptides were determined by matching the MS/MS spectrum to a known database of peptide spectra using search algorithms, such as SEQUEST. This algorithm was set to perform no-enzyme (unconstrained) and uninterrupted searches of the IPI (International Protein Index) and/or NR databases[10].

Multivariate pattern recognition analysis

The regions that were significantly different from the six groups were used to perform multivariate pattern recognition including unsupervised principal component analysis and supervised orthogonal projections to latent structures-discriminant analysis (OPLS-DA). These analyses were used to determine the distributions and find the metabolic difference between the six groups using MetaboAnalyst 3.0. The OPLS-DA models were cross-validated using a 10-fold method with unit variance scaling. The R2 values were used to evaluate the fit of the OPLS-DA models, and Q2 was used to assess their predictive ability. To determine which coefficients contributed to sample clustering, we used MATLAB to generate coefficient plots, which were colour-coded with the absolute value of the correlation coefficients.

Results

Significant m/z values discovered among saliva samples from the six groups

The mass spectra of the extracted peptide samples in the six groups are shown in Figure 2. The peaks in the saliva peptidome fingerprints were characterised by finding the maximum intensity within a certain m/z range. Then, the peaks among the mass spectra were quantified and compared. It was found that 182 peptides mass peaks were significantly different ($P < 0.05$) when the six groups were compared by ANOVA.

Multivariate statistical analysis

The macroscopic results were shown in Figure 3 for the 182-peptide data set, after applying OPLS-DA. Groups T1, T2, T3, T4, T5 and T6 were located in six directions, showing that metabolic changes could be detected after AOO. Biomarker profiles varied over time: levels of the metabolites first decreased then increased in T2, peaked in T4 and then declined until T6 and showed a tendency to approach T1. The R2 and Q2 values of component 1 were 0.96 and 0.89 respectively, and of component 2 were 0.98 and 0.96 respectively. The results of multiple comparisons between the two groups were shown in Table 1.

Table 2 Significant ($P < 0.01$) m/z values identified in multiple comparisons between groups (A vs B).

m/z value	*P*-value	-log10(P)	FDR P [1]	The results of post-hoc tests: Group (A vs B)[2]
2775.33	3.32E-17	16.479	5.26E-16	T1 vs T2; T1 vs T3; T4 vs T1; T1 vs T5; T1 vs T6; T2 vs T3; T4 vs T2; T2 vs T5; T2 vs T6; T4 vs T3; T4 vs T5; T4 vs T6
1692.92	7.82E-16	15.107	8.95E-15	T4 vs T1; T5 vs T1; T6 vs T1; T4 vs T2; T5 vs T2; T6 vs T2; T4 vs T3; T5 vs T3; T6 vs T3; T5 vs T4; T6 vs T4
3052.02	9.24E-14	13.034	7.32E-13	T1 vs T2; T1 vs T3; T1 vs T4; T1 vs T5; T1 vs T6; T2 vs T3; T2 vs T4; T5 vs T2; T5 vs T3; T5 vs T4; T5 vs T6
2027.72	6.56E-11	10.183	2.76E-10	T1 vs T2; T3 vs T1; T1 vs T4; T1 vs T5; T1 vs T6; T3 vs T2; T2 vs T4; T2 vs T5; T2 vs T6; T3 vs T4; T3 vs T5; T3 vs T6; T4 vs T5
2690.66	7.31E-06	5.1361	1.38E-05	T1 vs T2; T1 vs T3; T1 vs T4; T1 vs T5; T1 vs T6; T3 vs T2; T4 vs T2; T3 vs T6; T4 vs T5; T4 vs T6
1864.17	7.58E-05	4.1203	1.24E-04	T1 vs T3; T1 vsT2; T1 vs T4; T2 vs T3; T2 vs T4; T2 vs T6; T5 vs T3; T6 vs T3; T5 vs T4
1473.44	0.0030018	2.5226	0.003659	T6 vs T1; T6 vs T2; T6 vs T3; T6 vs T4; T6 vs T5

[1] The FDR P is the *P*-value adjusted using the false discovery rate.
[2] The results showed which groups were significantly different from each other.

Predictions of peptide identities were made as follows (Table 2):

- The peptides identified at 2691.6 Da were predicted to be vitamin D-binding protein precursors
- Those identified at 3052.02 and 2775.33 Da were predicted to be Apolipoprotein A-I precursors (APOA1).
- The peptides at 1473.44 and 1692.92 Da were predicted to be Isoform 1 of fibrinogen alpha chain precursor (FGA).
- The peptides at 1864.17 Da and 2027.72 Da were predicted to be complement component 3 (C3).
- Changes to mass peaks with time after corticotomy were found to be as follows (Fig 4):
- The 2691.6 Da peptide (predicted to be vitamin D-binding protein precursors) increased in T2 then fell to preoperative levels.
- The mass peaks of 3052.02 Da and 2775.33 Da peptides (predicted to be APOA1) increased sharply in T2 then decreased.
- The mass peaks of 1473.44 and 1692.92 Da peptides (predicted to be Isoform 1 of FGA) decreased at first, then increased with time.
- The 1864.17 and 2027.72 Da peptides (predicted to be C3) decreased in T2; then gradually increased, peaked in T5 and declined in T6.

Discussion

The rationale for the resent study was first the lack of information on the biomarkers present in RAP, and secondly that most previous studies investigating the AOO mechanism were invasive and animal-based[5,6,8]. Therefore, we used MALDI TOF MS as a new, non-invasive, simple and low-cost tool to identify candidate biomarkers responsible for physiological or pathological conditions of accelerating tooth movement. The present study found significant differences in mass spectra peak intensities among the six different groups according to AOO treatment duration. It indicated that this current method could be used to analyse peptide profile features of patients facilitated by AOO treatments.

The AOO procedure involves production of various factors, such as hormones and cytokines, which can stimulate alveolar bone metabolism and thereby accelerate tooth movement. We wanted to find candidate biomarkers involved in both alveolar bone remodelling and the inflammatory reaction. An effective biomarker should be measurable in an easily-accessible body fluid, such as serum, urine, or saliva[11,12]. Since saliva collection is non-invasive, simple and inexpensive, and contains abundant proteins and peptides, it is increasingly

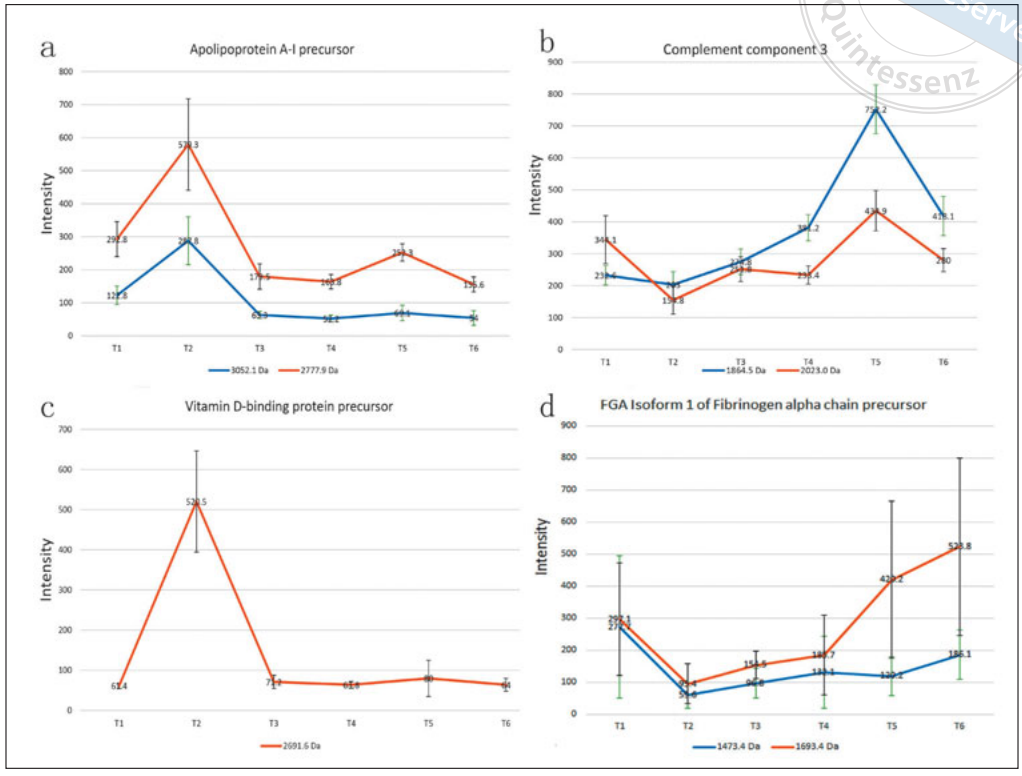

Fig 4 Time line charts showed the trend in peak intensity from the six groups: a) 3052.02 Da and 2775.33 Da predicted to be the Apolipoprotein A-I precursor; b) 1864.17 Da and 2027.72 Da predicted to be complement component 3; c) 2691.6 Da predicted to be the vitamin D-binding protein precursor; d) 1473.44 Da and 1692.92 Da predicted to be Isoform 1 of the fibrinogen alpha chain precursor.

used for the analysis of oral and systemic conditions[13]. The current study included Class III surgical patients who had undergone AOO treatment to examine saliva samples.

The MALDI TOF MS-based proteomic methods and WCX magnetic beads were sensitive enough to detect a large mass range, and the mass spectra are easy to interpret[9]. The effectiveness of this method has been confirmed in many peptide profile identification studies[9,13].

The peptides identifications have led to interesting speculations in this study. The mass peaks of 3052.02 Da and 2775.33 Da peptides predicted to be the Apolipoprotein A-I precursor (APOA1) increased sharply during the week after corticotomy then decreased. In a previous study, APOA1 was the most prominent protein that significantly decreased in osteopenic femurs. In addition, it appeared to be the most significant variations of proteins in patients with osteopenia and osteoarthritis[14]. Some studies reported that corticotomy induced a localised increase in turnover of alveolar spongiosa and the acceleration of a physiologic demineralisation and remineralisation process, reversible osteopenia[6]. There is sufficient evidence to suggest that APOA1 plays an important role in reversible osteopenia, which is involved in accelerated tooth movement. Blair demonstrated in APOA1 deficient mice reduced bone formation rate and reduced bone mass (osteopenia) while osteoclast function was unaffected; APOA1 deficiency reflects reduced bone synthesis while osteoclast differentiation seems to be unaffected. In aggregate, the Blair data presented suggest that APOA1 deficiency can be used as a biomarker for reduced bone density and/or reduced bone anabolic function relative to bone catabolic function[15]. Increased APOA1 at 1 week suggests the osteogenesis-osteoclasis balance favoured bone formation; decreased APOA1 at 2 weeks suggests the osteogenesis-osteoclasis balance favoured bone resorption, i.e. osteopenia. Rationale for this interpretation is that alveolar corticotomy increases tissue strain – the immediate biological response in order to reduce tissue strain is by increasing bone strength by way of bone modelling apposition, especially woven bone formation[16]. Woo successfully used an apolipoprotein A-1 mimetic peptide (L-4F) in a murine lupus erythematosus model of accelerated atherosclerosis to combat decreased bone mineral density[17]. The apolipoprotein A-1 mimetic peptide significantly prevented overall bone loss and additional osteopenic manifestations. The authors suggested that L-4F could potentially be utilised to target inflammatory lipids, and as a result, limit the

progression of inflammation. In our study, APOA1 suppressed over 6 months post-corticotomy suggests an extended inflammation mediated osteopenia (RAP) process. Caput compared human bone tissues with and without osteopenia in sexa- and septa-genarians[14]. Bone mineral density differences in the femur heads removed during hip replacement surgery with and without osteopenic hips averaged 18%. The excised femur heads were subjected to bone proteome analysis. Difference gel electrophoresis (DIGE) technology was used to determine proteins that were differentially expressed. Standard liquid chromatography mass spectrometry demonstrated that apolipoprotein A–I was the most prominent protein that significantly decreased (23-fold) in the osteopenic femur heads.

Ehrnthaller et al reported that complement component 3 (C3) may play a role in bone development and that C3 deficiency in mice affected fracture healing[18]. In our study, the 1864.17 Da and 2027.72 Da peptides, predicted to be C3, gradually increased after corticotomy, peaked in T5 and declined in the 6 months after the procedure. Our results suggested that activation of C3 might be crucial for bone metabolism during orthodontic tooth movement. The 2691.6 Da peptide, which is predicted to be a vitamin D-binding protein precursor, is thought to bind to vitamin D and its plasma metabolites and transport them to target tissues[19]. Previous findings suggest that local application of 1.25-dihydroxyvitamin D3 acts directly to increase osteoclast number and to potentiate bone resorption after mechanical stimuli[20]. In rats receiving 1.25-dihydroxyvitamin D3 every 3 days, tooth movement significantly increased[21]. Local application of 1.25-dihydroxyvitamin D3 enhanced the reestablishment of supporting tissue, especially the alveolar bone of teeth, after orthodontic treatment[22].

As these biomarkers were present in substantial quantities during AOO-induced tooth movement, they could be useful for analysing the relationship between biomarkers and bone remodelling, and also for accelerating tooth movement during AOO treatment.

The results of this study should be interpreted with the following limitations in mind. These differential expression patterns of significantly altered proteins may originate from the bone metabolism or tooth movement, or corticotomy-associated inflammation. In addition, the analysis of saliva is inherently challenging because peptides in saliva may be posttranslationally modified, or could be proteolytic fragments of related proteins[23]. Therefore, a peptide sequence does not necessarily define a single protein. In subsequent work, we hope to establish a concise monitoring model for AOO procedures and confirm the specific peptides found in this study.

Conclusion

Our study suggested that the salivary protein profiles, analysed by magnetic bead-based MALDI-TOF MS, changed with AOO treatment duration. This method provides a new tool for investigating accelerated tooth movement in AOO procedures and also explores the factors responsible for the regional acceleratory phenomenon (RAP). This could be used as a preliminary base on which to accelerate the progress of orthodontic treatment.

Conflicts of interest

The authors reported no conflicts of interest related to this study.

Author contribution

Dr Jia Qi WU performed the clinical procedures, prepared and revised the manuscript; Dr Jiu Hui JIANG conceived and designed the study; Drs Li XU, Cheng LIANG and Xiu Jing WANG performed the clinical procedures; Dr YunYang BAI performed the clinical procedures, reviewed and revised the manuscript.

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