Apolipoprotein E is an effective biomarker for orthodontic tooth movement in patients treated with transmission straight wire appliances

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Introduction: Orthodontic tooth movement (OTM) is the core component of orthodontic treatment and is increasingly popular for treating malocclusions. In this study, we aimed to investigate the role of apolipoprotein E (ApoE) in OTM. **Methods:** Thirty patients treated with transmission straight wire technology were selected and longitudinally tracked at 2 different stages of orthodontic treatment (initial 2 months and 12 months of orthodontic treatment). Total saliva was collected and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Western blotting was used to detect the difference in ApoE expression in the saliva samples of the 2 groups. The expression of ApoE was further verified by immunohistochemical staining in a mouse model of tooth movement. **Results:** The results of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry showed significant differences in the components of the salivary peptides in the 2 groups and peptides with a molecular weight of 2010.7 Da were predicted to be ApoE by database analysis. Western blotting further verified a significant difference in the expression of salivary ApoE in the 2 groups. In addition, an OTM model was successfully constructed in mice. The immunohistochemical staining results showed that ApoE expression significantly increased after force loading in the OTM model. **Conclusions:** This study indicated that ApoE participated in and played a role during OTM in patients treated with transmission straight wire technology. This relationship might be related to alveolar bone reconstruction and root resorption. The results provide new ideas for research on the mechanism of tooth movement using precision medicine based on saliva detection. (Am J Orthod Dentofacial Orthop 2021;■:■-■)

Malocclusion is listed as 1 of the 3 major oral diseases by the World Health Organization and significantly influences patients' oral functions, esthetics, psychology, and other aspects.[1,2] An increasing number of patients with malocclusion seek orthodontic treatment. Orthodontic tooth movement (OTM) is the core component of orthodontic treatment.[3] OTM is a process of dynamic reconstruction of local periodontal tissue under mechanical force, which enables teeth to move to a new position in the alveolar bone through bone absorption at the pressure side and bone deposition at the tension side.[4] Alveolar bone remodeling in the OTM process is research hotspot.[5-7] How mechanical force acts on the periodontal microenvironment to induce alveolar bone metabolism is a key issue that needs to be fully elucidated.

Saliva is rich in proteins, peptides, small molecules, and other compounds.[8] Salivary-based proteomics or peptidomics has become a focus of research in recent years because of its noninvasive, convenient, and low-cost properties.[9,10] In recent years, highly convenient and sensitive mass spectrometry (MS) technology has identified various proteins in saliva.[11] Alveolar bone remodeling, root resorption, and tissue inflammation occur following tooth movement.[12,13] These physiological or pathologic conditions are usually accompanied by changes in biomarkers.[14] Some studies have shown that

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the process of orthodontic treatment is related to changes in saliva components, most of which are related to the physical properties of the saliva, microbial activity, metal ions released by orthodontic appliances, or factors involved in the pain of patients after orthodontic treatment.[15-17] However, few studies have directly explored the relationship between OTM and biomarker expression.

Our research group previously reported in a cross-sectional study the significant difference in the composition of salivary peptides in patients at different stages during orthodontic treatment through MS analysis[18] and predicted 1 of the biomarkers to be apolipoprotein E (ApoE). However, the results need further verification. In previous studies related to ApoE, the focus has primarily been on its relationship with lipid metabolism. In recent years, research has indicated that ApoE also plays an important role in bone metabolism.[19,20] Alveolar bone remodeling during orthodontic tooth movement (OTM) is an important aspect of bone metabolism. However, the association between ApoE and alveolar bone remodeling has not been previously reported.

Therefore, this study aimed to further explore the change in biomarkers and the possible mechanism in the process of orthodontic treatment, and the role of ApoE was investigated in this longitudinal study. In addition, we hoped to provide new ideas and methods involving saliva-based precision medicine for the detection of OTM-related biomarkers that underlie alveolar bone remodeling during orthodontic treatment.

MATERIAL AND METHODS

The subjects of this study were patients admitted to the orthodontic department of Peking University School and Hospital of Stomatology. Subjects with systemic diseases, dental caries, gingivitis or periodontitis, oral mucosal diseases, and oral cancer were excluded. A total of 14 men and 16 women were included in this study. The mean age was 16.07 ± 1.86 years.

All 30 patients had similar malocclusion. They were diagnosed as Angle's Class II malocclusion with the complaint of mouth protrusion and crowding. The crowding degree was mild to moderate (within 6 mm) for maxillary and mandibular arches. The anchorage was moderate and/or strong for the maxillary arch and moderate for the mandibular arch.

A larger range of tooth movement and more active bone remodeling occurs in patients treated with tooth extraction than nonextraction. Therefore, patients treated with extraction were chosen for this study. The orthodontic treatment plan was to extract the maxillary first premolars and mandibular second premolars. Transmission straight wire appliances (Shinye, China), developed by Lin Jiuxiang from Peking University School and Hospital of Stomatology, were used for the patients. The overall process for the orthodontic treatment was as follows: the patients came for the first visit 2 weeks after teeth extraction, and the appliances were bonded during this visit. Next, the patients visited once a month. The assessment of patients' oral hygiene status is provided in Supplementary Tables I and II. Light orthodontic force was used for each patient during the orthodontic treatment.

The orthodontic procedures could be divided into the following 3 phases. Phase I: a 0.016-in Australian wire with helical loops on both sides between maxillary lateral incisors and canines was used. Nickel-titanium (NiTi) round archwires could be used as auxiliary archwires when crowding existed among the maxillary anterior teeth. Backward bending was performed in the mesial position of maxillary first molars. Appropriate Class II elastic traction between the maxillary and mandibular arches was used to reduce the anterior overjet and the extraction space by retracting the maxillary 6 anterior teeth together. Phase II: a 0.016-in Australian wire was continuously used. In addition, Class I elastic traction, combined with Class II elastic tractions if necessary, were applied to close the remaining extraction space. The molar relationship was also adjusted to neutral. Phase III: The archwires were changed in sequence from NiTi round wires to NiTi square wires to stainless steel archwires to adjust the tip and torque of the teeth. The above procedures were controlled as similarly as possible for the patients, including the same order of archwire replacement and the same visit interval.

This study was approved by the biomedical ethics committee of Peking University School and Hospital of Stomatology (approval no. SSIRB-201311103; IRB12014). Informed consent was signed by the patients and/or their guardians.

Saliva samples were collected twice, the first at 2 months and the second at 12 months after orthodontic treatment. The 2-month collection was used as a baseline control of the tooth movement in this longitudinal study. At about 2 months of orthodontic treatment, the early changes were relatively stable. In addition, the tooth movement was also activated at this time. Therefore, 2 months with mild tooth movement and 12 months when obvious tooth movement occurred were chosen as the 2-time points for MS analysis to investigate the change in salivary biomarkers during the process of OTM. The saliva collection procedures conformed with our previous study.[18] The protein concentration was determined, and the supernatant was maintained at −80°C for further analysis.

After suspension shaking and elution, the magnetic beads were separated from the protein, and the eluted peptide sample was transferred to a clean 0.5 mL sample tube for MS analysis.

Five μL of alpha-cyano-4-hydroxycinnamic acid substrate solution (0.4 g/L, soluble in acetone and ethanol) and 0.8-1.2 μL of eluent were mixed. The 0.8-1.2 μL mixture was then coated on the metal target plate and dried at room temperature. Finally, the prepared samples were analyzed with the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), peptides in the range of 1000-10,000 Da molecular weight were collected, and the laser energy was used 400 times. Peptide fingerprints were obtained by 50 single MS signal scans.

Western blotting was used to detect ApoE expression in the saliva. Total protein in the saliva samples was extracted, and the relative concentration was measured by a bicinchoninic acid kit (Kangwei Century Biotechnology, Beijing, China). The protein was heated at 95°C for 5 minutes for full denaturation. The protein samples underwent sodium dodecyl sulfate-polyacrylamide gel electrophoresis to fully separate the target band and were wet transferred to a polyvinylidene fluoride membrane. Primary antibody (anti-ApoE; Shanghai ABtech Antibiotics Co, LTD) was used at a ratio of 1:500 for 1 hour after blocking. After overnight incubation with primary antibody, triple buffer saline was used for rinsing. Then, a secondary antibody (anti-GAPDH; Cell Signaling Technology, Boston, Mass) was applied for incubation and exposure. Total proteins were blotted using coomassie blue staining. In addition, the ratio of target band intensities to total protein intensity was used for Western blot normalization.

To further confirm and double-check the results histologically, a mouse animal model of tooth movement was used. Male C57BL/6 mice were used to establish the OTM model. The NiTi tension spring was bonded with flowing resin from the first molar on the maxillary right side to the incisor of the mice. The stretch force was approximately 30 g of force, according to previous studies.[21,22] The contralateral teeth were used as controls, and data were recorded at 7 days and 14 days from the date of force loading.

After a certain experimental period, the animals were killed by excessive pentobarbital. The maxilla was separated, fixed, decalcified and dehydrated, and then embedded in paraffin. Continuous sections of 4 μm were made perpendicular to the tooth axis, and the upper one-third slices of each sample were taken for histological experiments. The paraffin sections were dewaxed, hydrated and incubated at room temperature with 3% hydrogen peroxide for 30 minutes. Then antigen repair was performed with mixed enzyme solution (0.125% trypsin and 10 mg/mL protease K), and the sections were incubated again at room temperature for 30 minutes. The tissues were sealed with 3% bovine serum albumin at room temperature for 30 minutes. Primary antibody (anti-ApoE, diluted to a certain proportion with antibody diluent) was followed for 4°C overnight. The hypersensitive 2-step method kit corresponding to the primary antibody species was applied. The horseradish peroxidase-labeled secondary antibody was added, and the samples were incubated in a water bath at 37°C for 30 minutes. Following the above steps, diaminobenzidine chromogenic staining, hematoxylin staining, differentiation, gradient alcohol dehydration, xylene transparency, and sealing were performed sequentially. Finally, the sections were ready for observation and photograph.

Statistical analysis

In this study, the effect size for the primary outcome (ApoE) was expected to be 126.43, which was the average change of peak intensity determined from our preliminary experiments. Based on these parameters, the calculated sample size for the paired samples estimated that 13 subjects would be required to achieve at least 80% power with a type II error rate (β) of 0.2 and α of 5%. A paired t test was used to determine the differences in peptides levels in the saliva samples between the 2-time points. Student t test and 1-way analysis of variance were used for the semiquantitative analysis of ApoE expression in immunohistochemical results. The BioExplorer statistical package was used for data analysis, and $P < 0.05$ indicated a statistical difference.

RESULTS

To explore the change in relevant biomarkers during orthodontic treatment, we performed MALDI-TOF-MS of the 30 included subjects at 2-time points to obtain the mass spectra of the extracted samples. The mass peaks of saliva proteins were characterized in each patient by presenting the intensity within a particular mass-to-charge ratio (m:z) range. Then, the mass spectra peaks were quantified and compared. The molecular weight range of the detected peptides was 1000-10000 Da. As a result, an average of 128 protein mass peaks was found. Three peaks (2010.7, 1526.1, and 2326.3 Da) were significantly different between the 2 groups (Table). Figure 1 shows the comparison of the 3 peaks. The peak intensity of 1526.1 Da was

Table. Significant ($P < 0.05$) *m/z* values distinguishing between 2-mo and 12-mo groups

Mean m/z value	P	*Tendency**
2010.7	0.001	↑†
1526.1	0.037	↓‡
2326.3	0.046	↑†

*Tendency: m/z intensity between the 2 groups; †higher peak intensity in the 12-mo group than the 2-mo group; ‡lower peak intensity in the 12-mo group than in the 2-mo group.

lower in the 12-month group than in the 2-month group. The peak intensities of 2010.7 Da and 2326.3 Da were higher in the 12-month group than in the 2-month group. Moreover, the peptides 2010.7 Da and 1526.1 Da were chosen to establish a scatter fitting graph, which showed a differentiated scope of the 2 groups (Fig 2).

The 2010.7 Da peak, which was the most significantly different factor between the 2 groups was successfully identified and predicted to be Apo E, by matching the MS/MS spectrum to a known in the silico-generated database of peptide spectra and using supporting softwares analysis. To verify the expression level of the 2010.7 Da peak, we performed Western blotting to compare the Apo E levels between the 2 groups. The results showed that the salivary ApoE level was significantly higher in the 12-month group than in the 2-month group (Fig 3).

Thus, to further confirm the results histologically, a mouse OTM model was established in this study with force loading to the maxillary first molar. The immunohistochemical results showed that ApoE expression was significantly different at different loading time points (7 d and 14 d) (Fig 4). The semiquantification of ApoE-positive periodontal membrane cells at 2-time points in Figure 4, *B* and *C* showed the ApoE expression levels increased after force loading, suggesting that ApoE might be involved in tooth movement and alveolar bone remodeling under mechanical force stimulation. Compared with the nontooth movement control, there was a significant difference in the expression of ApoE both on the compression side and on the tension side at 7 days and 14 days after force loading. Moreover, the expression of ApoE on the compression side was higher than that on the tension side in the 7 d group, and the difference was statistically significant. However, there was no statistical significance in the expression of apoE between the compression side and tension side in the 14 d group. In addition, the expression of ApoE in the 14 d group was significantly higher than that in the 7 d group both on the compression and tension side, and the differences were statistically significant.

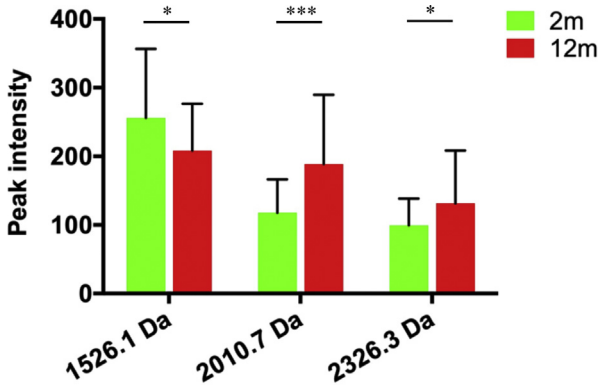

Fig 1. The comparison of the 3 significantly different peaks between the 2 groups. The peak intensity of 1526.1 Da was lower in the 12-mo group than in the 2-mo group. The peak intensities of 2010.7 Da and 2326.3 Da were higher in the 12-mo group than in the 2-mo group. *$P < 0.05$; ***$P < 0.001$.

DISCUSSION

Previous studies have suggested that OTM is caused by the local aseptic inflammatory response of periodontal tissues under orthodontic force.[23] Force loading could lead to upregulation of prostaglandin E, interleukin-1, tumor necrotic factor, and other inflammatory factors, inducing osteoclastic activity of local alveolar bone and ultimately OTM.[24-26] Mechanical force-induced alveolar bone remodeling is the core focus in the OTM process. The periodontal membrane is located between the root and alveolar bone. During the process of OTM, periodontal ligament cells[27] are exposed to mechanical force and stimulate the expression of a series of chemotactic factors and inflammatory markers,[28] which are closely related to periodontal tissue reconstruction[29]; however, the specific mechanism is unclear.

Many studies have shown that various cytokines could be detected in the peripheral blood or gingival crevicular fluid of orthodontic patients and the periodontal ligaments of experimental animal models.[30] A biomarker is a signal that could be a hormone, cytokine, growth factor, inflammatory factor or any other factor associated with a particular condition.[31] Because of their specificity and sensitivity, biomarkers are important in the diagnosis of specific diseases or conditions. Effective biomarkers could be detected in body fluids, including serum, urine, or saliva.[32] Saliva has attracted the attention of scholars in recent years.[33] In recent years, highly convenient and sensitive MS techniques have identified various biomarkers in saliva.[34]

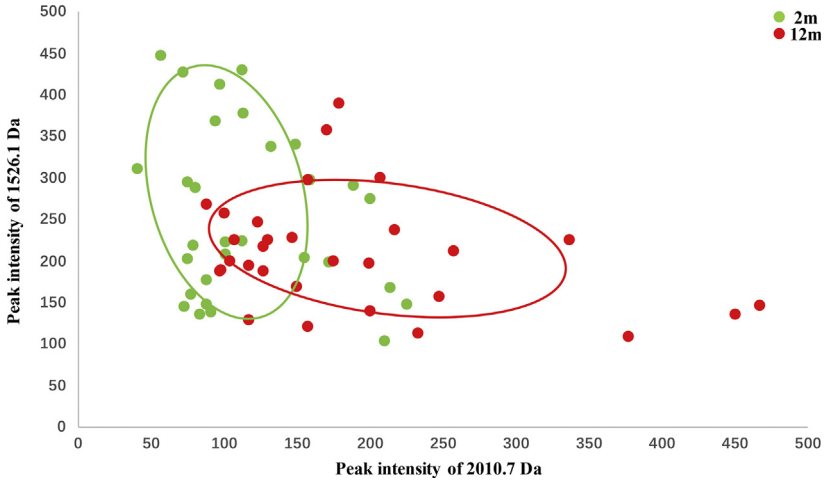

Fig 2. The scatter plots fitting the shape between the 2 groups using the peptides 2010.7 Da and 1526.1 Da. The figure shows a relatively well-separating effect.

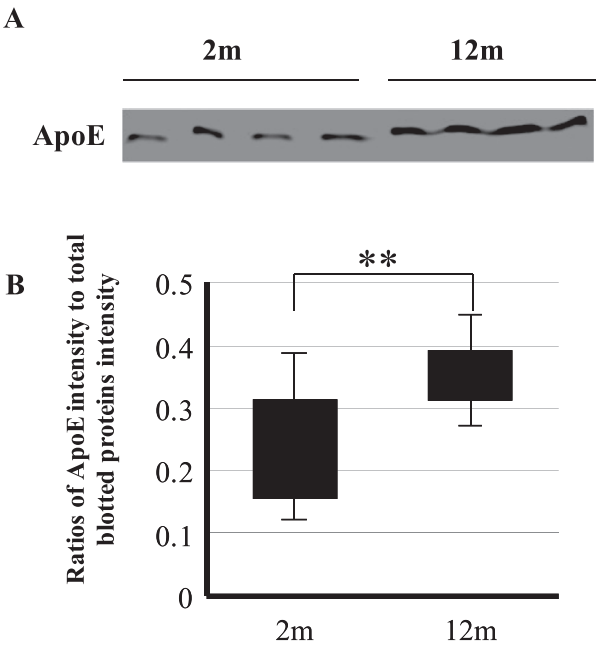

Fig 3. ApoE levels in the saliva samples from the 2 groups. **A,** The salivary ApoE expression was verified by western blotting. **B,** Normalized ApoE (ApoE relative to total blotted proteins) band intensity of the 2 groups. $^{**}P < 0.01$.

Some studies have shown that orthodontic treatment is related to changes in saliva components; however, biomarker information and the mechanism are still unclear.[35,36] This study detected the differences between 2 different time points during the orthodontic treatment.

ApoE is a 34 kDa glycoprotein, mainly produced by the liver and large phages, which is considered an important factor in hyperlipidemia and atherosclerosis, and *apoE* knockout mice show lipidemia and atherosclerosis.[37] Recent studies have indicated that ApoE not only plays an important role in lipid metabolism but also in bone metabolism.[19] Many reports have shown that the bone formation and bone mass of elderly *apoE* knockout mice are reduced, and some other studies have suggested that the bone mass of young *apoE* knockout mice is higher than that of young wild-type mice, and the influence of pathophysiological conditions such as hyperlipidemia, obesity, and renal insufficiency on bone was changed in knockout mice.[38] Other studies have shown that ApoE could promote the uptake of vitamin K generated by lipoprotein by osteoblasts by downregulating *p*53, which inhibited the apoptosis of osteoblasts and indirectly promoted bone formation.[39] In addition, ApoE was reported to downregulate the phosphorylation of extracellular signal-regulated kinase 1/2 and participated in bone metabolism,[20] and the extracellular signal-regulated kinase 1/2 pathway is related to the osteoclast differentiation process induced by receptor activator of nuclear factor kappa B ligand.[40]

Alveolar bone remodeling during OTM is an important aspect of bone metabolism. However, the association between ApoE and alveolar bone remodeling has not been previously reported. In our study, ApoE was significantly different during the OTM process based on the MS analysis, and the difference was further verified through western blotting. The results suggested that ApoE might be involved in tooth movement and alveolar

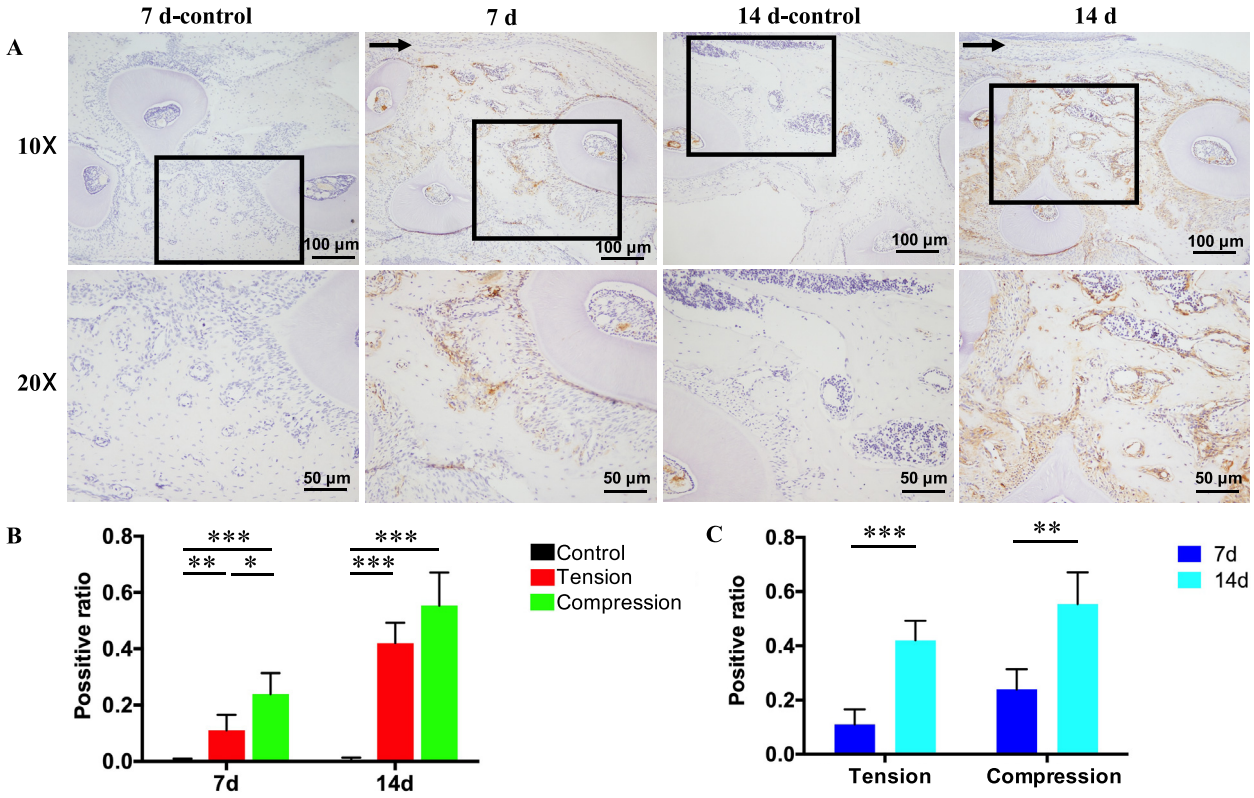

Fig 4. ApoE expression in mouse OTM model. **A,** Image of the immunohistochemical staining shows ApoE expression at different loading time points (7 d and 14 d). The *upper row* shows the staining results at 10 × magnification under a microscope; the lower row shows the 20 × magnification of the black square shown in the *upper row*. **B,** Semiquantification of ApoE-positive periodontal membrane cells at 2-time points. The expression of ApoE after force loading increased significantly compared with the nontooth movement control both on the compression side and the tension side. The expression of ApoE on the compression side was significantly higher than that on the tension side in the 7 d group. There was no significant difference between the compression side and the tension side in the 14 d group. **C,** Comparison of ApoE expression at 2-time points. The expression of ApoE in the 14 d group was significantly higher than that in the 7 d group both on the compression side and the tension side, and the difference was statistically significant. *Arrows* indicate the direction of tooth movement. n = 5. **P* <0.05; ***P* <0.01, ****P* <0.001.

bone remodeling. In the present longitudinal study, the tooth movement mode for each part of the treatment procedure among the patients was similar, ensuring the difference in treatment duration and the associated changes were the main factors indicative of different salivary protein expression.

Moreover, the immunohistochemical results in the mouse OTM model showed that ApoE expression was significantly different at different loading time points, which further confirmed the results histologically. Besides, the difference in the expression of ApoE on the compression side and the tension side may reflect the changes in the expression of ApoE in the osteogenesis of the tension side and the alveolar bone resorption of the compression side. The results could contribute to future in-depth mechanism studies.

Our study did not include the samples without tooth movement as a control for MS analysis, which was a limitation. In future work, we will design studies including patients at pretreatment and postorthodontic evaluation and long-term follow-up. In addition, a tooth movement model was constructed in mice in this study to simulate the clinical OTM. The time points of 7 d and 14 d were chosen following previous studies. However, the mouse OTM at 7 d and 14 d is not comparable to human tooth movement. The association between mouse OTM and human tooth movement during orthodontics requires further study.

CONCLUSIONS

1. Alveolar bone remodeling during OTM is an important aspect of bone metabolism, but no study has explored the correlation between ApoE and OTM-related alveolar bone remodeling. This study suggests that the expression of ApoE significantly increased with the progression of orthodontic treatment. In addition, the immunohistochemical results in the mouse OTM model further confirmed the results histologically, indicating that ApoE might be involved in alveolar bone remodeling during tooth movement.
2. This study helped elucidate the molecular biological mechanism of OTM and provided theoretical support and experimental basis for exploring the acceleration of OTM, shortening the course of orthodontic treatment, and alleviating the discomfort of patients with malocclusion during orthodontic treatment.
3. Precision medicine based on saliva detection to explore biomarkers related to OTM might be a new method to study this process.

AUTHOR CREDIT STATEMENT

Jieni Zhang contributed to conceptualization, formal analysis, investigation, and original draft preparation; Bohui Sun contributed to resources and data curation; Huaxiang Zhao contributed to resources and manuscript review and editing; Ting Zhang contributed to investigation; Danqing He contributed to resources and manuscript review and editing; Jiuxiang Lin contributed to conceptualization and supervision; and Feng Chen contributed to methodology and supervision.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ajodo.2020.08.020.

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Supplementary Table I. Distribution of plaque index and gingival index values in the 2-mo and 12-mo groups

		Index score			
Index	*Group*	*0*	*1*	*2*	*3*
Plaque index	2-mo	3 (10)	20 (67)	7 (23)	0 (0)
Plaque index	12-mo	5 (17)	21 (70)	4 (13)	0 (0)
Gingival index	2-mo	3 (10)	23 (77)	4 (13)	0 (0)
Gingival index	12-mo	5 (17)	22 (73)	3 (10)	0 (0)

Note. Values are n (%).

Supplementary Table II. Plaque index and gingival index values summarized by means and standard deviations for 2-mo and 12-mo groups

Index	*2-mo*	*12-mo*	P
Plaque index	1.33 ± 0.57	0.97 ± 0.56	0.26
Gingival index	1.03 ± 0.49	0.93 ± 0.52	0.45