Biochemical markers of bone metabolism during early orthodontic tooth movement with aligners

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ABSTRACT

Objective: To evaluate the expression of receptor activator of nuclear factor-kappa ligand (RANKL), osteoprotegerin (OPG), osteopontin (OPN), interleukin 1β (IL-1β), and transforming growth factor ß1 (TGF-ß1) in the gingival crevicular fluid (GCF) of teeth subjected to orthodontic forces released by aligners.

Materials and Methods: A total of 10 healthy, adult patients were selected to participate in this split-mouth study. The treatment plan was designed to obtain only one movement with the first aligner: distalization of a second molar. GCF samples were obtained from pressure and tension sites of the test tooth and from the mesiobuccal and distobuccal sites of the control tooth. The GCF sample volumes were measured using a Periotron 8000. Levels of TGF-β, IL-1β, RANKL, OPG, and OPN were measured by enzyme-linked immunosorbent assay.

Results: IL-1β showed a significant increase at the pressure sites after 1 week and 3 weeks with respect to baseline ($P < .05$) compared with control sites. The kinetics of TGF-1β and OPN were characterized by a significant increase at the tension sites of the test teeth ($P < .05$) after 3 weeks from the application of orthodontic force. The RANKL level was significantly increased at pressure and tension sites after 1 hour and after 1 week from the application of the orthodontic forces ($P = .023$ and $P = .043$, respectively).

Conclusions: An increased concentration of bone modeling and remodeling mediators at the pressure sites (IL-1β, RANKL) and tension sites (TGF-1β, OPN) was observed. These scenarios are compatible with previous in vivo and in vitro studies investigating the biological effects of orthodontic tooth movement. (*Angle Orthod.* 2017;87:74–81)

KEY WORDS: Aligners; Invisalign; RANKL; Interleukin 1β; Gingival crevicular fluid

INTRODUCTION

The application of an orthodontic force produces a tissue reaction resulting from the perturbation generated by the orthodontic appliance and the modeling and remodeling of the alveolar bone.[1]

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Aligners provide intermittent orthodontic forces and, despite their spread among the orthodontic community, there are no studies describing the bone metabolism induced by this kind of appliance. Kuncio et al.[2] suggested that teeth moved with aligners did not undergo the typical stages of movement, as described by Krishnan and Davidovitch,[3] because of the intermittent forces applied by the aligners. However, light, continuous forces seem to be perceived as intermittent forces by the periodontium due to its viscoelastic nature[4] and orthodontic intermittent forces are able to produce orthodontic tooth movement (OTM) with less cell damage in the periodontium.[5]

The inflammatory response to OTM is associated with the production and release of a variety of cytokines. Some of these cytokines, including transforming growth factor beta (TGF- β) and interleukin 1 beta (IL-1β), stimulate osteoclast differentiation, function, and survival, contributing to the bone remodeling mechanism and tooth movement.[6,7] Stimulation of the

bone cells is mediated by several factors, including a member of the tumor necrosis factor (TNF) ligand and receptor superfamilies, including the receptor activator of nuclear factor kappa B ligand (RANKL), the receptor activator of nuclear factor kappa B (RANK), and osteoprotegerin (OPG).[8–11] Osteopontin (OPN) is another protein that has been linked to bone resorption via promotion of osteoclast adhesion to the osseous matrix.[12]

Gingival crevicular fluid (GCF) is the medium used to detect molecules involved in bone modeling and remodeling processes during OTM.[13–17] However, no data are available in relation to aligner orthodontics.

This is the reason we decided to conduct a prospective, split-mouth study to measure the concentration of IL-1β, RANKL, OPG, OPN, and TGF β1 in GCF volumes obtained from teeth subjected to early orthodontic forces in patients undergoing orthodontic treatment with aligners.

MATERIALS AND METHODS

Subjects

Participants were recruited from the Department of Orthodontics of the CIR Dental School of the University of Torino during the period March–September 2015. Eligibility for inclusion in the study were (1) >18 years of age, (2) good health status, (3) previous extraction of maxillary third molars, (4) bilateral end-to-end Class II molar relationship, (5) mesodivergent craniofacial typology (SpP-GoGn angle = 25° ± 6°), (6) good control of oral hygiene, (7) no anti-inflammatory or antibiotic therapy in the previous 6 months. Exclusion criteria were (1) smoking habit and (2) signs of gingivitis or periodontitis.

A total of 10 healthy, adult patients for which orthodontic treatment with aligners was programmed (5 men, 5 women, age [mean ± SD] 22.3 ± 3.3) were selected to participate in this split-mouth study. For each subject, one second molar was randomly selected as a test tooth, and the contralateral molar served as control.

Informed consent was obtained from each subject. The study was conducted in accordance with the Declaration of Helsinki, and the research protocol was approved by the local ethics board (No. 3732015, CIR Dental School, Turin University).

Intervention

The standardized orthodontic intervention was represented by the Invisalign (Align Technology, San Jose, Calif) orthodontic appliance. Using Clin-Check software (Align Technology) the treatment was planned to distalize only a randomly selected second molar in isolation at the beginning of treatment. All the treatment plans were designed by the same operator and staging was set at 0.25 mm per aligner.[16] The study lasted for 3 weeks, during which the investigated subjects used only the first aligner planned with only one movement, that is, distalization of one second molar. A recent study[17] demonstrated that forces released by the aligner decay over time without any evidence of affecting the efficiency of tooth movement.

Attachments were not programmed for the first aligner. In accordance with the existing literature, the initial distalizing force was 1 N.[18] Two weeks before beginning the study, all the subjects underwent a supragingival prophylaxis and were given oral hygiene instructions to follow at home to eliminate inflammation. The study lasted for 21 days and involved three visits. At the first visit (baseline), the Silness and Loe Plaque (PI) Index, Lobene Modified Gingival Index (GI), and Bleeding on Probing (BOP) Index were recorded for the control and for the test molar. The BOP Index was evaluated after GCF sampling to avoid possible variations. GCF samples were thus obtained from the mesiobuccal and distobuccal aspects of both the test tooth and control tooth. The Invisalign aligner was then delivered, and proper instructions were provided the patient. Compliance indicators embedded in each posterior segment of the maxillary aligner were monitored during the study.[19] Clinical measurements were then repeated after 1 hour, 7 days, and 21 days after appliance delivery. GCF samples were obtained from the mesiobuccal (tension) site and distobuccal (pressure) site of the test and control molars at each visit. All samples were collected by the same operator (EFG).

CGF Sampling

GCF samples were collected 1 hour before appliance placement (T0), 1 hour after aligner delivery (T1), 7 days after (T2), and 21 days after (T3). Days 7 and 21 were chosen for GCF sampling because day 7 is the turnover time for enzymes and indirect resorption processes start on day 21.[20] Two GCF samples for each site were collected using PerioPaper strips (Oraflow Inc, Plainview, NY). Teeth were isolated with cotton rolls, cleaned of plaque deposits, and dried gently with air before paper strips were applied 1 mm subgingivally for 30 seconds. The volume of the sample on the paper strips was measured using a calibrated Periotron 8000 (OraFlow). The readings from the Periotron were converted to an actual volume (microliters) by reference to the standard curve calibrated with human serum.[21] The GCF absorbed on each paper point was diluted with 250 μL of sterile

phosphate-buffered saline (pH 7.4), centrifuged (13,000 g at 4°C for 15 minutes), and stored at −80°C until analysis.[22] The Periotron was always calibrated before GCF sampling.[21]

Biochemical Analysis

The biologist at the Biochemistry Lab of the Department of Orthodontics of the University of Torino performing GCF cytokine measurement was blinded to the patients' clinical details. After thawing at room temperature, stored GCF samples were assayed in duplicate for IL-1β (Diaclone SAS, Besancon, France), RANKL (Biomedica Immunoassay GmbH & Co, Vienna, Austria), OPG (Biomedica), OPN (IBL International, Hamburg GmbH, Germany), and TGF β1 (Diaclone) by enzyme-linked immunosorbent assay according to the manufacturer's instructions. Optical density (OD) was measured on a plate reader using 450 nm wavelength. Since OD is directly proportional to cytokine concentration, a standard curve generated from the OD values of standards provided by the manufacturer was used to determine cytokine concentration. The given concentrations from duplicate measurements were combined, and mean cytokine concentration was converted into total cytokine amount per 250 μL of sample. Finally, cytokine concentration in the GCF was calculated by dividing the total cytokine amount by GCF volume (μL) adsorbed on a Perio-Paper strip.

Statistical Analysis

Statistical analysis was performed using MedCalc software, version 12.7.0.0 (MedCalc, Ostend, Belgium). Cytokine levels were expressed as mean ± standard deviation (SD). Data normality was checked using the D'Agostino-Pearson test. In order to analyze longitudinal variations of cytokine levels (kinetics) within groups (intragroup analysis) and to compare those variations between groups (intergroup analysis), repeated measures analysis of variance (ANOVA) was performed. Grouping variables were appliance of orthodontic force (test sites vs control sites) and GCF sampling site (mesiobuccal sites vs distobuccal sites). Paired t-tests were used to compare cytokine levels at the different time points in which samples were taken from the patients, between and within groups. A P value of $<.05$ was considered statistically significant.

RESULTS

No significant differences were detected for the considered periodontal indexes (PI, GI, or BOP) at any time point for either test or control teeth (PI tests vs controls, $P = .259$; GI tests vs controls, $P = .259$; BOP tests vs controls, $P = .500$). According to Tuncay et al.,[19] compliance indicators revealed a mean wearing time of about 22 hours per day.

While GCF volumes did not differ either between or within test sites or control sites at any time point (Table 1), significant differences were revealed for several markers when comparing test vs control teeth (Tables 2 and 3; Figure 1).

The ANOVA test for repeated measures resulted in significant variations in cytokine kinetics within and between the considered groups, respectively (Table 4).

When considering pressure and tension sampling sites, IL-1β showed a significant increase at the pressure sites after 1 week and after 3 weeks with respect to baseline ($P < .05$; Figure 2). Intergroup longitudinal analysis revealed significant differences at both pressure ($P = .007$) and tension sites ($P = .03$; Table 5).

The kinetics of TGF-1β and OPN were characterized by a significant increase at the tension sites of the test teeth ($P < .05$) after 3 weeks from the application of orthodontic force. The intergroup longitudinal analysis revealed significant differences at tension sites ($P = .003$ and $P = .001$, respectively; Table 5). OPG was significantly decreased at the pressure and tension tests sites after 1 week and 3 weeks with respect to baseline ($P = .018$ and .047, respectively).

RANKL level was significantly increased at pressure and tension sites after 1 hour and 1 week from the application of orthodontic force ($P = .023$ and .043, respectively). The intergroup longitudinal analysis conducted about RANKL concentration revealed significant differences at the pressure sites ($P = .014$; Table 5).

DISCUSSION

In recent years, aligner orthodontics has grown rapidly as an increasing number of patients have sought an esthetic and comfortable alternative to fixed appliances.[23] The results of the present study represent the first attempt to clarify the biological mechanisms behind aligner orthodontics.

The existing literature demonstrated that interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α) are proinflammatory cytokines involved in alveolar bone remodeling following force application.[10,24] While GCF volumes did not differ between or within test sites and control sites as previously described in the literature,[13] interestingly, a significant increase of IL-1β in the GCF samples of test teeth with respect to those obtained from the control teeth was observed in this study. Furthermore, when considering GCF samples obtained from pressure and tension sites, an increased level of

Table 1. GCF Volumes in Tension (M) and Compression Sites (D) (Test Sites) and Mesiobuccal (M) and Distobuccal Sites (D) (Control Sites)

	Time Point	Test Sites M	Test Sites D	Control Sites M	Control Sites D
GCF[a]					
(μL)	T0	0.17 ± 0.07[b]	0.17 ± 0.08	0.19 ± 0.08	0.16 ± 0.08
GCF	T1	0.16 ± 0.06	0.23 ± 0.10	0.18 ± 0.09	0.18 ± 0.08
GCF	T2	0.18 ± 0.07	0.18 ± 0.07	0.18 ± 0.10	0.16 ± 0.10
GCF	T3	0.15 ± 0.05	0.19 ± 0.08	0.15 ± 0.06	0.15 ± 0.07

[a] GCF indicates gingival crevicular fluid.
[b] All variables are expressed as mean ± standard deviation.

1β was evident at the pressure sites. This increase was statistically significant with respect to levels detected at the tension sites. Thus, our results are in agreement with previous in vivo studies demonstrating an association between GCF IL-1β levels and OTM.[3,6]

TGF-1β is one of the key cytokines with pleiotropic properties that has both proinflammatory and anti-inflammatory features in the regulation of the inflammatory infiltrate.[24] After 3 weeks of aligner therapy, our results showed an increased level of this cytokine at the tension sites of the test teeth. TGF-1β plays a role in bone destruction; however, an increased concentration of this marker has been demonstrated at both tension and compression sites, revealing a complex role of this mediator.[25]

Table 2. Cytokine Comparison Between Test Sites and Control Sites According to Time Point Sampling

Cytokine	Time Point	Test Sites	Control Sites	P
IL-1β[a] (pg/μL)	T0	34.9 ± 17.5[b]	36.4 ± 13.2	.630
IL-1β	T1	32.7 ± 23.0	29.7 ± 13.3	.649
IL-1β	T2	45.7 ± 30.9	35.9 ± 11.2	.204
IL-1β	T3	62.7 ± 32.9	31.6 ± 11.4	.002
TGF-β1 (pg/μL)	T0	62.0 ± 26.1	79.7 ± 25.7	.189
TGF-β1	T1	80.9 ± 26.6	76.5 ± 28.3	.425
TGF-β1	T2	74.8 ± 25.5	85.8 ± 26.3	.224
TGF-β1	T3	118.7 ± 43.8	84.2 ± 38.4	.085
OPG (pg/μL)	T0	4.2 ± 2.0	4.4 ± 3.5	.810
OPG	T1	3.4 ± 2.5	4.7 ± 3.1	.129
OPG	T2	2.2 ± 1.9	4.3 ± 2.7	.011
OPG	T3	2.5 ± 2.0	5.6 ± 4.5	.039
OPN (ng/μL)	T0	28.1 ± 15.5	31.2 ± 15.7	.426
OPN	T1	35.6 ± 19.9	26.2 ± 13.6	.177
OPN	T2	35.2 ± 18.5	32.9 ± 14.5	.695
OPN	T3	46.0 ± 22.7	31.3 ± 14.7	.054
RANKL (pg/μL)	T0	0.5 ± 0.4	0.7 ± 0.5	.218
RANKL	T1	1.2 ± 0.8	0.8 ± 0.7	.004
RANKL	T2	1.3 ± 0.9	0.8 ± 0.4	.066
RANKL	T3	2.5 ± 2.2	0.9 ± 0.7	.041

[a] IL-1β indicates interleukin-1 beta; OPG, osteoprotegerin; OPN, osteopontin; RANKL, receptor activator of nuclear factor kappa B ligand; TGF-β1, transforming growth factor-beta 1.
[b] All variables are expressed as mean ± standard deviation. P values were evaluated by paired t-tests.

Table 3. Cytokine Levels Compared Between Different Time Points According to Test Sites and Control Sites

	IL-1β[a]	TGF-β1	OPG	OPN	RANKL
Test sites (pg/μL)					
T0 vs T1	0.759	0.124	0.389	0.063	0.008
T0 vs T2	0.244	0.271	0.007	0.128	0.023
T0 vs T3	0.011	0.001	0.047	<0.001	<0.001
T1 vs T2	0.203	0.511	0.151	0.927	0.893
T1 vs T3	0.007	0.004	0.184	0.021	<0.001
T2 vs T3	0.007	0.011	0.490	0.013	<0.001
Control sites (pg/μL)					
T0 vs T1	0.056	0.579	0.512	0.150	0.598
T0 vs T2	0.861	0.088	0.855	0.666	0.201
T0 vs T3	0.218	0.618	0.247	0.497	0.977
T1 vs T2	0.100	0.091	0.512	0.101	0.799
T1 vs T3	0.659	0.521	0.392	0.166	0.286
T2 vs T3	0.248	0.858	0.312	0.755	0.682

[a] IL-1β indicated interleukin-1 beta; OPG, osteoprotegerin; OPN, osteopontin; RANKL, receptor activator of nuclear factor kappa B ligand; TGF-β1, transforming growth factor-beta 1. P values were evaluated by paired t-tests.

Bone remodeling is controlled by a balance between RANK-RANKL binding and OPG production.[25,26] Numerous studies have demonstrated that the RANK signaling pathway is crucial for differentiating and activating osteoclasts.[26] Osteoprotegerin (OPG) is expressed in osteoblastic cells as well as RANKL, and it is a decoy receptor produced by osteoblastic cells, which compete with RANK for RANKL binding.[25]

Increased RANKL and decreased OPG secretions in human PDL cells were demonstrated as a consequence of compressive forces, and these

Table 4. Intragroup and Intergroup Longitudinal Analysis in Test Sites and Control Sites

	Cytokine Levels Variation[b]	Cytokine Kinetics Comparison[b]
IL-1β[a] (pg/μL)		
Test sites	P = .011	P = .008
Control sites	P = .190	
TGF-β1 (pg/μL)		
Test sites	P < .001	P = .001
Control sites	P = .499	
OPG (pg/μL)		
Test sites	P = .044	P = .078
Control sites	P = .373	
OPN (pg/μL)		
Test sites	P = .001	P = .013
Control sites	P = .355	
RANKL (pg/μL)		
Test sites	P = .001	P = .032
Control sites	P = .279	

[a] IL-1β indicates interleukin-1 beta; OPG, osteoprotegerin; OPN, osteopontin; RANKL, receptor activator of nuclear factor kappa B ligand; TGF-β1, transforming growth factor-beta 1.
[b] Cytokine levels variation within groups (intragroup analysis) and cytokine kinetics comparison between groups (intergroup analysis) were evaluated by repeated measures ANOVA.
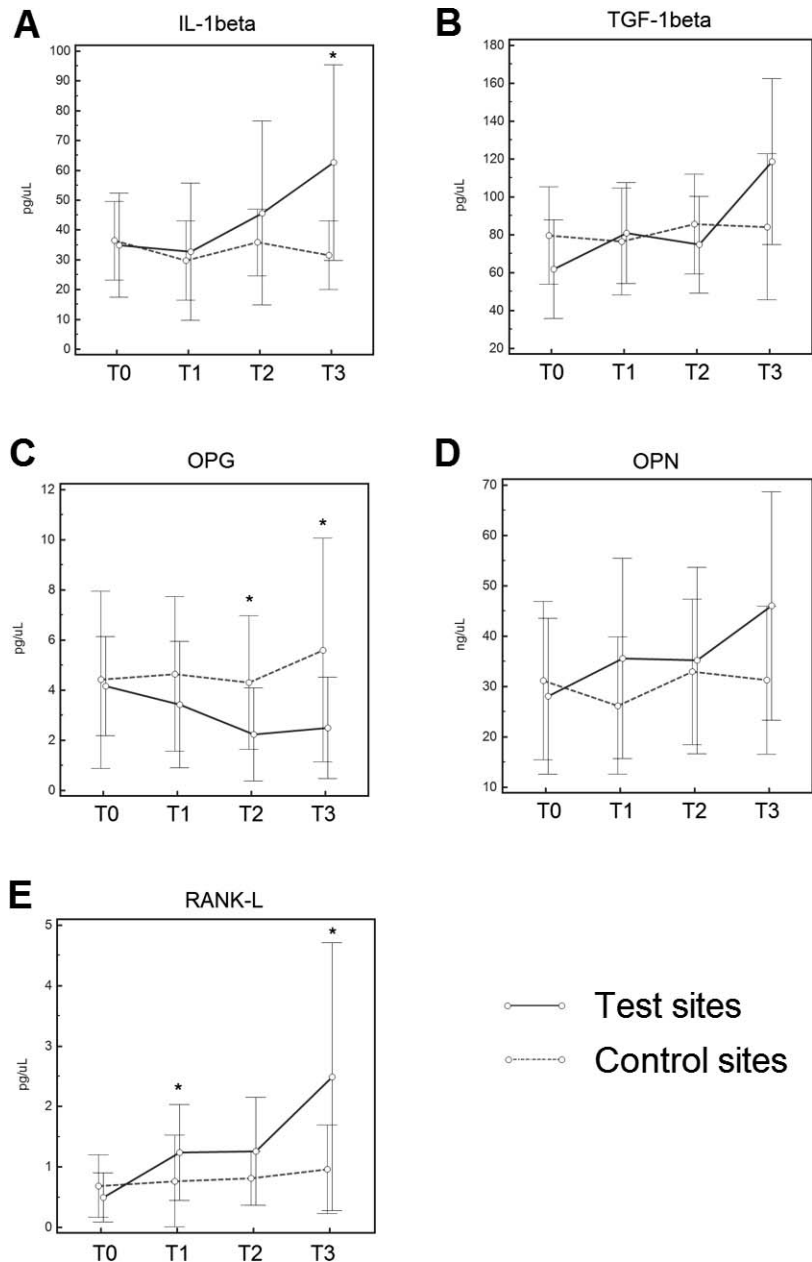
Figure 1. IL-1β (A), TGF-1β (B), OPG (C), OPN (D), and RANKL (E) kinetics in test sites and control sites. * Difference between test sites and control sites at a corresponding time point is statistically significant ($P < .05$). P values were evaluated by paired t-tests. Cytokine values are depicted as mean ± standard deviation.

responses were time- and force-magnitude dependent.[8] Our results confirm these observations: at the compression site, we observed a significant increase of RANKL and a significant decrease of OP. Barbieri et al.[15] described a significant difference for RANK concentration after 1 day of force delivery (elastic separator) at pressure sites, differences that were not detected after 7 days, concluding that RANK may have a short- term role in bone changes. Our results demonstrated an increase of RANKL after 1 hour and

1 week of force delivery, confirming the role of the RANK/RANKL/OPG system in the early stages of orthodontic treatment. When comparing test sites vs control sites, we noted that a significantly increased concentration of RANKL in test sampling sites was still evident after 3 weeks of force delivery.

Osteopontin (OPN), one of the major noncollagenous bone proteins, acts not only as a trigger for osteoblast early differentiation, but it can also inhibit osteoclastic activity.[26] According to these observations,
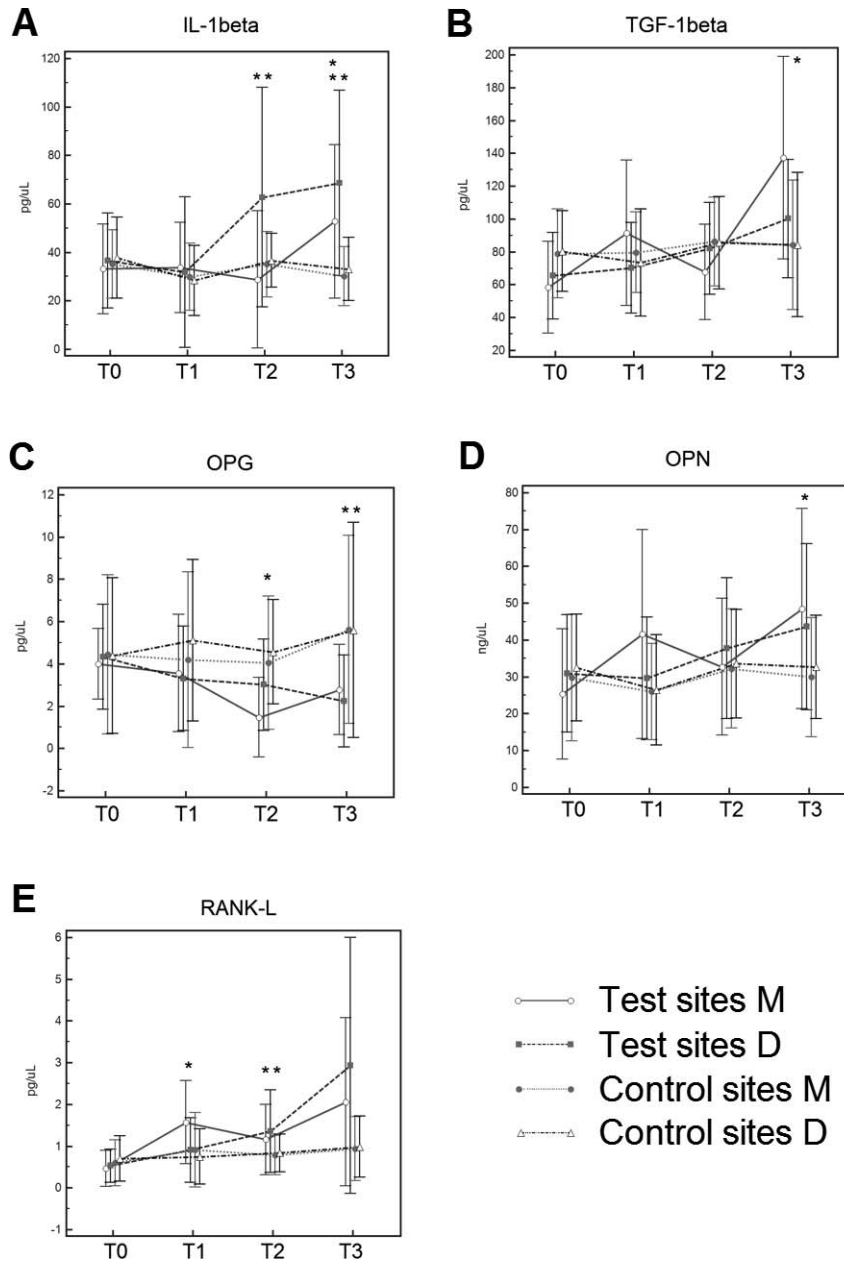
Figure 2. IL-1β (A), TGF-1β (B), OPG (C), OPN (D), and RANKL (E) kinetics according to tension (M) and compression sites (D) (test sites) and mesiobuccal (M) and distobuccal sites (D) (control sites). * Difference between test sites M and control sites M at corresponding time point is statistically significant (*P* < .05). *P* values were evaluated by paired *t*-tests. ** Difference between test sites D and control sites D at corresponding time point is statistically significant (*P* < .05). *P* values were evaluated by paired *t*-tests. Cytokine values are depicted as mean ± standard deviation.

results from the present study support a significant increase of OPN concentration in the GCF samples obtained from the tension sites of the test teeth after 3 weeks of force delivery.

Caution should be always taken when analyzing cytokines in GCF samples because of the possibility of increased secretion due to periodontal plaque-related inflammation. Although elevated levels of cytokines have been related to periodontal disease,

the increased concentrations of IL-1β and TNF-α family cytokines observed in this study are below those of patients with gingivitis or periodontitis. Furthermore, periodontal indexes did not change during the study period, indicating absence of periodontal inflammation. Therefore, the increased cytokines levels measured in this study are within the limits of an acceptable physiological response. The observed differences can be attributed solely to the

Table 5. Intergroup Analysis According to Sampling Sites

Cytokine	Group	Kinetics Comparison[b]	T0	T1	T2	T3
IL-1β [a] (pg/μL)	Test sites M vs test sites D	0.067	0.398	0.744	0.009	0.040
	Control sites M vs control sites D	0.855	0.546	0.433	0.570	0.310
	Test sites M vs control sites M	0.030	0.625	0.556	0.395	0.018
	Test sites D vs control sites D	0.007	0.810	0.673	0.036	0.003
TGF-β1 (pg/μL)	Test sites M vs test sites D	0.055	0.169	0.221	0.112	0.045
	Control sites M vs control sites D	0.805	0.343	0.127	0.896	0.989
	Test sites M vs control sites M	0.003	0.172	0.335	0.129	0.048
	Test sites D vs control sites D	0.214	0.228	0.681	0.677	0.375
OPG (pg/μL)	Test sites M vs test sites D	0.259	0.511	0.616	0.014	0.287
	Control sites M vs control sites D	0.850	0.927	0.580	0.433	0.976
	Test sites M vs control sites M	0.174	0.733	0.587	0.018	0.067
	Test sites D vs control sites D	0.154	0.952	0.112	0.058	0.047
OPN (pg/μL)	Test sites M vs test sites D	0.068	0.210	0.149	0.045	0.481
	Control sites M vs control sites D	0.954	0.162	0.827	0.706	0.244
	Test sites M vs control sites M	0.001	0.331	0.115	0.948	0.041
	Test sites D vs control sites D	0.392	0.715	0.537	0.492	0.158
	Test sites M vs test sites D	0.273	0.212	0.032	0.215	0.335
	Control sites M vs control sites D	0.605	0.343	0.305	0.506	0.604
	Test sites M vs control sites M	0.089	0.432	0.023	0.075	0.064
	Test sites D vs control sites D	0.014	0.327	0.209	0.043	0.073

[a] IL-1β indicates interleukin-1 beta; OPG, osteoprotegerin; OPN, osteopontin; RANKL, receptor activator of nuclear factor kappa B ligand; TGF-β1, transforming growth factor-beta 1.

[b] Kinetics comparison between groups (intergroup analysis) was performed by repeated measures ANOVA. *P* values between groups for each single time point were evaluated by paired *t*-tests.

induced orthodontic tooth movement. The use of the split-mouth design and the results achieved on the control sites support these considerations. When one considers the wide range of biological responses and interfering factors in both the PDL and GCF,[27] the number of subjects participating in this study may be a limiting factor.

A limitation of this study was the absence of an internal error method analysis for GCF sampling and quantification. To overcome this limitation, we calibrated the Periotron before each GCF sampling.[21]

In conclusion, aligners seem to be capable of inducing the same biological responses described for other appliances, at least in the very early stages of orthodontic treatment.

CONCLUSIONS

- Invisalign aligners release an initial force of about 1 N on distalizing a maxillary molar. This force delivery produces an increased concentration of bone modeling and remodeling mediators at both pressure sites (IL-1β, RANKL) and tension sites (TGF-1β, OPN).
- These scenarios are compatible with previous in vivo and in vitro studies investigating the biological effects of OTM.
- Further studies are required to elucidate aligners' OTM effects on longer observation periods.

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