ORIGINAL ARTICLE

Effect of use of antiseptics and fluorides during orthodontic treatment on working properties of NiTi archwires in levelling dental arches

A randomized controlled trial

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Abstract

Purpose The goal was to investigate whether the application of antiseptics and fluorides during orthodontic treatment affects the biomechanics of the levelling of dental arches by changing the working properties of nickel–titanium (NiTi) archwires.

Methods The sample consisted of 60 individuals aged 12–22 years (53% females). There were 20 individuals in each experimental group: (I) individuals conducted regular oral hygiene, (II) individuals used a high concentration of fluorides for intensive prophylaxis for the first month; and (III) individuals used chlorhexidine in the same manner. NiTi alloy archwires (dimensions 0.508×0.508 mm) were analyzed 3 months after intraoral exposure and compared to as-received NiTi wires. Elastic modulus, yield strength, springback ratio, and modulus of resilience were calculated. Dimensions of the dental arches were analyzed when NiTi alloy (T1) was placed intraorally and after 3 months (T2). Change was quantified as the difference in dimensions (T2–T1). Anterior width-to-length ratio was used as a measure for dental arch shape.

Results Intraoral exposure reduced elastic modulus, yield strength, springback ratio, modulus of resilience, loading, and unloading forces of NiTi wires (*p*≤ 0.021). Chlorhexidine mouthwash and gel with high concentration of fluorides did not change these properties more than saliva with regular hygiene. The amount of change of dental arch shape in the maxilla and mandible did not differ significantly between the experimental groups.

Conclusion Using antiseptics or a high concentration of fluorides during orthodontic treatment does not significantly affect the mechanical properties of NiTi wires and would not have clinical implications in changing orthodontic biomechanics.

Keywords Dental biofilms · Enamel demineralization · Oral antiseptics · Biomechanics · Elasticity

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Wirkung der Anwendung von Antiseptika und Fluoriden während der kieferorthopädischen Behandlung auf die Arbeitseigenschaften von NiTi-Bögen in der Nivellierung von Zahnbögen

Eine randomisierte, kontrollierte Studie

Zusammenfassung

Absicht Das Ziel der Studie war es zu untersuchen, ob die Anwendung von Antiseptika und Fluoriden während der kieferorthopädischen Behandlung die Biomechanik der Nivellierung der Zahnreihen beeinflusst, indem die Arbeitseigenschaften von NiTi(Nickel-Titan)-Bögen verändert werden.

Methode Die Stichprobe bestand aus 60 Personen im Alter von 12–22 Jahren, von denen 53% weiblich waren. Es gab jeweils 20 Personen in jeder experimentellen Gruppe: (I) Personen, die regelmäßige Mundhygiene durchführten, (II) Personen, die im ersten Monat eine hohe Konzentration von Fluoriden für eine intensive Prophylaxe verwendeten, und (III) Personen, die Chlorhexidin auf die gleiche Weise verwendeten. NiTi-Legierungsbögen (Abmessungen 0,508×0,508mm) wurden nach 3 Monaten intraoraler Exposition analysiert und mit ursprünglich erhaltenen NiTi-Bögen verglichen. Elastizitätsmodul, Streckgrenze, Rückfederungsfaktor und Resilienzmodul wurden berechnet. Die Dimensionen der Zahnbögen wurden analysiert, als die NiTi-Legierung intraoral eingesetzt wurde (T1) und nach 3 Monaten (T2). Die Änderung wurde als Unterschied in den Dimensionen (T2–T1) quantifiziert. Das Verhältnis von Breite zu Länge im vorderen Bereich wurde als Maß für die Form des Zahnbogens verwendet.

Ergebnis Die Ergebnisse zeigten, dass die intraorale Exposition das Elastizitätsmodul, die Streckgrenze, den Rückfederungsfaktor, das Resilienzmodul sowie die Belastungs- und Entlastungskräfte der NiTi-Bögen reduzierte (*p*≤ 0,021). Chlorhexidin-Mundspülung und Gel mit hoher Fluoridkonzentration veränderten diese Eigenschaften nicht mehr als Speichel bei regelmäßiger Hygiene. Die Veränderung der Form des Zahnbogens im Ober- und Unterkiefer unterschied sich nicht signifikant zwischen den experimentellen Gruppen.

Schlussfolgerung Die Anwendung von Antiseptika oder einer hohen Konzentration von Fluoriden während der kieferorthopädischen Behandlung hat keine signifikanten Auswirkungen auf die mechanischen Eigenschaften von NiTi-Bögen und keine klinischen Auswirkungen auf die Veränderung der kieferorthopädischen Biomechanik.

Schlüsselwörter Dentale Biofilme · Demineralisierung von Zahnschmelz · Orale Antiseptika · Biomechanik · Elastizität

Introduction

After many years of development and improvements in orthodontic treatment, certain fundamental aspects are important for successful outcome of orthodontic treatment. These include orthodontic biomechanics and the dental materials which comprise active and passive orthodontic elements.

Nickel–titanium (NiTi) wires have low stiffness and good springback, responding elastically to loading and return to their original shape when unloaded, with only low forces acting [\[1,](#page-8-0) [2\]](#page-8-1). Low stiffness is characterized by a low Young's modulus of elasticity (E), while yield strength (YS) is the maximum stress at which a material begins to deform plastically. The combination of E and YS is used to characterize flexibility as a response to loading by deformation without fracture, and resilience as the ability of an archwire to absorb energy during straining while being engaged in the brackets and to release energy during unloading while regaining its original shape. NiTi archwires are active elements that can generate a constant and light force during unloading. Unloading forces are key in orthodontics because they stimulate bone remodeling mediated by the tooth roots and periodontal ligaments. The direction of tooth movement is conditioned by the shape of the archwire ("archwire driven") and by the alveolar bone substrate, i.e., the alveolar envelope.

Clinical implications associated with corrosion of orthodontic appliances in the oral cavity include changes in archwire properties, e.g., roughness, friction, hardness, stiffness, flexibility, resilience, and strength [\[2\]](#page-8-1). Dental biofilm control is an ongoing struggle in dentistry and it has been confirmed that biofilm accumulation during 1 month of exposure in the oral cavity can reduce the unloading forces of NiTi archwires [\[3\]](#page-8-2).

Orthodontists are trying to combat the most common side effect of orthodontic treatment, which is a significantly increased risk for developing enamel demineralization and gingival inflammation caused by poor biofilm control, as well as oral mucosal lesions [\[4,](#page-9-0) [5\]](#page-9-1). To counteract these side effects, it is advisable to prescribe fluorides for the enamel and antiseptics for the soft tissues $[6-8]$ $[6-8]$.

Fluorides and antiseptics may also promote corrosion of NiTi archwires by changing their working properties, thus, interfering with the desired tooth movement and levelling of the dental arches $[9-11]$ $[9-11]$. However, the majority of existing evidence derives from in vitro research, which poses two problems—oversimplifying the simulation of the oral

environment and exaggerating the effects of the tested substances [\[9\]](#page-9-4).

Therefore, clinical trial and laboratory tests were combined in this research with the aim to investigate whether the application of antiseptics and fluorides during orthodontic treatment affects the biomechanics of dental arch levelling by altering the working properties of NiTi archwires. It was hypothesized that the intraoral application of antiseptics and high concentrations of fluorides would not significantly alter the flexibility and resilience of NiTi archwires and would not limit levelling of the dental arch.

Materials and methods

The local ethics committees approved the research and written informed consent was obtained from all subjects or their parents/legally authorized representatives for children under 18 years of age (Ethics Committee of the Clinical Hospital Centre Rijeka No. 2170-29-02/1-14-2 and Ethics Committee of the University of Rijeka, Faculty of Medicine No. 2170-24-01-14-05).

The sample for analysis consisted of 60 individuals aged 12–22 years (median 15; interquartile range 14–17 years);

Fig. 1 Consolidated Standards of Reporting Trials (CONSORT) flow diagram. *CHX* chlorhexidine treatment, *COVID* coronavirus disease 2019 **Abb. 1** CONSORT(Consolidated Standards of Reporting Trials)-Flussdiagramm. *CHX* Chlorhexidinbehandlung, *COVID* "coronavirus disease 2019"

53% of whom were females. A Consolidated Standards of Reporting Trials (CONSORT) flow diagram is shown in Fig. [1.](#page-2-0) Recruitment was initiated in November 2017, but the trial was stopped due to the coronavirus disease 2019 (COVID-19) pandemic in May 2020. All patients were undergoing orthodontic treatment with a $0.022''/0.56$ mm slot MBT fixed orthodontic appliance (American Orthodontics, Sheboygan, WI, US) at the University Dental Clinic in Rijeka, Croatia. There were 20 individuals in each of three parallel experimental group: (I) individuals conducting regular oral hygiene, (II) individuals using a fluoride gel for intensive prophylaxis for 1 month, and (III) individuals using chlorhexidine digluconate mouthwash in the same manner. Individuals were allocated to groups according to a randomization plan generated at [http://www.randomization.com.](http://www.randomization.com)

A NiTi alloy archwire (dimensions $0.020 \times 0.020''/0.508$ × 0.508mm; BioForce PLUS, Dentsply GAC International, Islandia, NY, USA) was placed intraorally after completion of the alignment phase of orthodontic treatment (6 months using NiTi archwires $0.014''/0.35$ mm and $0.016 \times 0.022''/0.41 \times 0.56$ mm) and left in place for 90 days. The alloy composition was $wt\%(Ni) = 50.4\%$ and $wt\%$ (Ti) = 49.6%.

Charters technique of brushing teeth was demonstrated to all subjects and they were instructed to brush their teeth for 2min three times a day. The first group was a control group and included subjects conducting regular oral hygiene, commonly recommended for orthodontic patients (toothbrush + toothpaste with a low fluoride concentration of 1450 ppm) throughout the study duration of 3 months. The second and third groups used adjuvant oral hygiene products during the first month: one group brushed their teeth three times per day without toothpaste and used a 0.12% chlorhexidine digluconate mouthwash (Curasept ADS 212, Curaden, Saronno, Italy) twice a day, while the other group brushed their teeth once a day without toothpaste and twice using a gel containing 6150 ppm of sodium fluoride (Mirafluor-K-gel, Hager Werken, Duisburg, Germany) for 30 days. For the next 2 months subjects performed standard hygiene (Charter's technique, 3 times per day for 2min with a toothpaste containing a low concentration of 1450 ppm fluoride).

A minimum sample size of 16 participants in each group was calculated as necessary if a change in arch shape (premolar width-to-length ratio) of 0.15 in one experimental group and 0.05 in the other group with a standard deviation (SD) of 0.10 in both groups is anticipated, with a power of 80% and significance of α = 0.05. For analysis of the mechanical characteristics, a sample size of 6 samples in each group was calculated to be sufficient to detect differences in Young modulus of 1.5 considering a standard deviation of 0.9.

Thus, archwires were collected from 18 participants and compared with as-received NiTi alloy wires. Six specimens from each experimental group, each 2.5 cm long, were used for mechanical three-point bending test in a thermal chamber at 37 °C on a universal machine Instron Model 1125/5500 (Instron, Binghamton, NY, USA). Each specimen was deflected to 3.1mm and then dropped to zero deflection at a crosshead speed of 0.0167mm/s. Force (N) and deflection (mm) were recorded every 5ms for each specimen during loading and unloading, using Texture Exponent software (Stable Micro System, Godalming, UK). Force–deflection curves were generated from these data. The elastic modulus (E), yield strength (YS), springback ratio as measures of flexibility (YS/E), and the modulus of resilience (YS²/2E) were calculated. The surface of representative specimens was analyzed with a JSM-7800F field emission scanning electron microscope (FE-SEM; JEOL, Tokyo, Japan) using a secondary electron detector with an electron beam accelerating voltage of 10 kV and a working distance of 10mm.

Plaster casts of the dental arches were made and analyzed before (T1) and after (T2 after 90 days) NiTi alloy archwire 0.508×0.508 mm were placed intraorally. Sliding caliper and Korkhaus caliper (Dentaurum GmbH, Ispringen, Germany) were used for measuring arch dimensions. Changes were quantified as the difference in dimensions (T2–T1) of anterior (premolar) and posterior (molar) arch width, and anterior (premolar) arch length in the maxilla and mandible. The following were measured: premolar arch width as the distance between the lower-most points of the transverse fissure of the maxillary first premolars, molar arch width as the distance between the point of intersection of the transverse fissure with the buccal fissure of the maxillary first permanent molar, and anterior arch length as a perpendicular from the most anterior labial surface of the central incisors to the connecting line of the reference points of premolar arch width. In the mandible, the vestibular contact points between the first and second premolar were used for the premolar width, and for the molar width the tip of the mediobuccal cusp of the mandibular first permanent molar were used. Changes were quantified as the difference in dimensions (T2–T1) in premolar and molar width of the dental arch, and lower premolar depth, separately for the maxilla and mandible. Premolar widthto-length ratio was used as a measure of the dental arch shape (a higher value indicated a more rectangular shape of the dental arch [wider and shorter] and a lower value indicated a more tapered or ovoid shape of the dental arch [narrower and elongated]). Persons who analyzed the plaster casts and mechanical properties of the archwires were blinded regarding group affiliations.

Normality of the data distribution was tested using the Shapiro–Wilk test. Statistical analyses included analysis of

a Kruskal–Wallis test

AS arithmetic mean, *SD* standard deviation, *Med* median, *IQR* interquartile range, *Min* minimum, *Max* maximum, *CHX* chlorhexidine

Fig. 2 Scanning electron microscope images (4000 × magnification) of as-received NiTi archwire sample and after exposure to experimental conditions. *CHX* chlorhexidine treatment

Abb. 2 Rasterelektronenmikroskopbilder (Vergr. 4000:1) von NiTi-Bogenproben vor und nach Exposition gegenüber experimentellen Bedingungen. *CHX* Chlorhexidinbehandlung

variance and Student–Newman–Keuls post hoc test for the archwire's working properties, while Kruskal–Wallis and Mann–Whitney tests with Bonferroni correction were used to assess arch shape changes. The Wilcoxon test was used to compare arch dimensions before and after 3 months. Commercial statistical software SPSS version 22.0 (IBM Corp., Armonk, NY, USA) was used and a significance level was preset to $p < 0.05$.

Results

Baseline characteristics

Baseline demographic and clinical characteristics of each group are shown in Table [1.](#page-4-0) Age distribution of men and women and premolar width–length ratio did not differ significantly between the groups.

Surface morphology

SEM images of the surface of the NiTi wires showing roughness and porosity in the as-received archwires and in the three experimental groups are presented in Fig. [2.](#page-4-1) Corrosion resulted in a smoothening of the surface under all conditions, which is due to a dissolution of surface peaks. However, the samples exposed to the fluoride medium demonstrated the highest number of surface defects.

Elastic properties

Intraoral exposure reduced the elastic properties of the NiTi wires ($p \le 0.021$), but fluorides and antiseptics did not change the properties more than saliva combined with regular oral hygiene (Figs. [3,](#page-5-0) [4,](#page-6-0) and [5\)](#page-7-0). Findings were the same for all measured loading and unloading forces at any deflection and also for the elastic modulus, yield strength, springback ratio, and modulus of resilience.

Changes in dental arch shape

Maxillary premolar length increased significantly in the control and in the group using antiseptics ($p \le 0.025$) with medians of 0.5 and 1mm, respectively. Maxillary premolar width increased only in the group using antiseptics (median = 0.5 mm; $p = 0.008$). There was a decrease in the **Fig. 3** Comparison of loading and unloading forces between experimental groups and unexposed archwires. *Asterisks* indicate the amounts of deflection in which the experimental groups differ significantly from the unexposed NiTi archwire. *CHX* chlorhexidine treatment **Abb. 3** Vergleich der Be- und Entlastungskräfte zwischen den experimentellen Gruppen und den nicht exponierten Bögen. *Sternchen* zeigen die Abweichungen an, in denen sich die experimentellen Gruppen signifikant von der unbelasteten Ni-Ti-Bogenprobe unterscheiden. *CHX* Chlorhexidinbehandlung

width–length ratio in the maxilla (median $= -0.02$ in the group using antiseptics and –0.06 in the control group), with the dental arch becoming more tapered (more in controls) or ovoid (more in the group using antiseptics). When the changes in the dimensions and shape of the maxillary arch $(\Delta T2-T1)$ were compared between the experimental groups, there were no statistically significant differences (Table [2,](#page-7-1) Fig. [6\)](#page-8-3).

There was a significant increase in premolar mandibular width in each of the three groups ($p \le 0.008$) with a median change of 1mm. Mandibular premolar length did not change significantly in any of the groups. Width–length ratio in the mandible mostly increased in the group using fluoride (median 0.08 ; $p = 0.023$). In the control group and in the group using antiseptics, mandibular width–length ratio also increased a median of 0.07 and 0.01, respectively, i.e., it reshaped to a more rectangular form. There were no significant differences in the amount of change of arch dimensions and shape in the mandible $(\Delta T2-T1)$ between the three experimental groups.

Discussion

The present research implies that application of fluorides and antiseptics during orthodontic treatment to control dental biofilm and enamel demineralization causes minor changes in the clinical performance of NiTi archwires.

Microstructure, pH of saliva, and the presence of fluorides cannot be acknowledged as possible reasons of corrosion of NiTi alloy, although published results vary greatly [\[12\]](#page-9-6). Qualitative surface analysis of the wires in the present research also showed a damaged surface after the use of high concentration fluoride gel, although our previous quantitative analysis implied that corrosion probably dissolves peaks, thus, smoothing the surface intraorally [\[13\]](#page-9-7). Intraoral corrosion seems inevitable. The presence of pre-existing defects on the surface of NiTi archwires can be the predilected locations for corrosion, while a titanium oxide layer protects against corrosion [\[14\]](#page-9-8). Longer in vivo expo-sure (2 months) decreased the corrosion susceptibility [\[15\]](#page-9-9). However, fluorides had a tendency to dissolve the titanium oxide layer causing localized, pitting corrosion [\[16\]](#page-9-10). The nickel ions are mostly released by corrosion during the first 3 days after which the corrosion tendency decreased [\[11\]](#page-9-5).

Our previously published results revealed that general corrosion is the predominant type of corrosion of intraoral aging of NiTi wires, but localized corrosion may also occur [\[13\]](#page-9-7). Corrosion behavior is similar when fluorides and antiseptics are used, but their use did not additionally increase the corrosion rate. Furthermore, surface roughness did not change significantly. The present research demonstrates that NiTi archwires after intraoral exposure under the influence of saliva and regular oral hygiene were less elastic and produced lower forces than as-received, unused archwires. Similar results were found in in vitro studies [\[17–](#page-9-11)[19\]](#page-9-12). The load–deflection properties of archwires are among the most important parameters in determining the biological response during tooth movement [\[20\]](#page-9-13). A threepoint bending test used in this research is the gold standard to assess the elastic properties of orthodontic archwire materials. Forces generated by orthodontic appliances are difficult to measure intraorally which is why elastic properties were measured on wires after removal from the mouth. This laboratory testing was able to measure unloading forces for different deflections which are equivalent to the resulting tooth displacement under clinical conditions.

Fig. 4 Comparison of Young's modulus of elasticity and yield strength (YS) in loading and unloading between experimental groups and unexposed archwires. *Horizontal lines* connect groups that differ significantly. *CHX* chlorhexidine treatment

Abb. 4 Vergleich des Elastizitätsmoduls und der Streckgrenze (YS) bei Belastung und Entlastung zwischen den experimentellen Gruppen und nicht exponierten Bögen. *Horizontale Linien* verbinden Gruppen, die signifikant voneinander abweichen. *CHX* Chlorhexidinbehandlung

According to our research, although some in vitro studies claim that topical fluoride agents affected the mechanical properties of NiTi wires during unloading of stress [\[10,](#page-9-14) [21\]](#page-9-15), neither high concentration of fluorides nor daily use of a chlorhexidine mouthwash for 1 month during orthodontic treatment further impaired the elastic properties of the wires.

In addition, straining of NiTi wires in the oral environment in the presence of fluoride is followed by a significant risk for corrosion–induced fractures [\[22,](#page-9-16) [23\]](#page-9-17). Earlier research reported no significant reduction in the load or deflection forces of NiTi wires in vitro [\[24\]](#page-9-18).

Previously, we reported that fluorides tended to reduce stiffness and hardness more than saliva or antiseptics, but this effect was not significant [\[13\]](#page-9-7). In that previous paper, only parts of the analysis of the mechanical and chemical properties of the NiTi wires were reported (roughness, friction, hardness, stiffness, and corrosion behavior). The present paper reports about the analysis of additional results from different samples from the same experiment (elasticity). In addition, clinical effects of the whole sample were calculated. Contrary to our findings, one clinical trial indicated that topical fluoride regimens reduced the unloading properties of NiTi wires in patients undergoing fixed orthodontic treatment, but only at higher deflections [\[25\]](#page-9-19).

The in vitro experiments revealed no significant effect of the chlorhexidine mouthwash on the roughness of NiTi archwires and on frictional resistance. Furthermore, no further deterioration in the elastic properties of the NiTi wires compared to the effects of saliva could be found [\[11,](#page-9-5) [26\]](#page-9-20). In contrast, another in vivo study found that both roughness and friction increased after the use of chlorhexidine mouthwash [\[27\]](#page-9-21). These opposite results may be due to the chlorhexidine molecules in the in vivo situation, where binding to the teeth and oral mucosa may have caused prolonged exposure. In vitro results suggested that a change in the pH of saliva had only minor influence on the hardness and modulus of elasticity of NiTi archwires [\[28\]](#page-9-22). In addition, consumption of soft drinks did not significantly lead to degradation of the physical and chemical properties of NiTi archwires [\[29\]](#page-9-23).

Fig. 5 Comparison of flexibility (springback ratio) and resilience in load and unload between experimental groups and unexposed archwires. *CHX* chlorhexidine treatment

Abb. 5 Vergleich der Flexibilität (Rückstellverhältnis) und der Resilienz bei Belastung und Entlastung zwischen den experimentellen Gruppen und nicht exponierten Bögen. *CHX* Chlorhexidinbehandlung

Corrosion does not necessarily lead to a major deterioration of the working properties of a NiTi alloy. A decrease in the elastic properties resulted in only smaller effects on dental arch levelling. For the assessment of dental arch shape changes, we defined dental arch shape as a ratio of premolar width and length to annul large interindividual variability in arch width and arch length. Changes in the shape of the dental arches were expected as these were defined by the original archwire form. As a consequence, the maxillary arch transformed towards a semi-ellipse, and the mandibular towards a parabola. This pattern was the same in all three experimental groups and was not significantly altered by fluorides or chlorhexidine application. To our knowledge, this is the first study that related laboratory tests of mechanical properties of orthodontic wires to clinical findings. The wires were analyzed after they had been

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Table 2 Comparison of changes in arch shape before and after experimental conditions
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a Kruskal–Wallis test

AS arithmetic mean, *SD* standard deviation, *Med* median, *IQR* interquartile range, *Min* minimum, *Max* maximum, *CHX* chlorhexidine treatment

Fig. 6 Comparison of changes in maxillary and mandibular dental arch shape between experimental groups. *CHX* chlorhexidine treatment **Abb. 6** Vergleich der Veränderungen der Form des Ober- und Unterkiefers zwischen den experimentellen Gruppen. *CHX* Chlorhexidinbehandlung

in the mouth for 3 months. This is usually the time when they are replaced with new wires as part of orthodontic treatment. Gel with high fluoride concentration is usually not used for more than 1 month. Chlorhexidine is also not used for a longer periods of time, as it causes discoloration of the teeth and tongue as well as a change in taste. Therefore, this experiment is a good approximation of the real and typical clinical situation. Alignment of the teeth had been done before the experiment started, but the leveling of the curve of Spee and also expansion of the arches was not completed. Thus, a possible association between any changes in wire properties due to experimental exposure to fluorides and antiseptics, and the resulting biomechanics could be established. The observed clinical effect is the result of the activity of the archwire in the mouth—it changes the shape of the dental arch. These were the advantages of this study. The limitations are that the extent of the levelling of the dental arches may have been determined by the alveolar envelope. However, NiTi arches do not control the vestibulo-oral position of the roots very well, but mostly tip the crown of the teeth. Therefore, we do not expect this to be a major limitation. Future studies should analyze the changes induced by thinner round wires in the initial alignment phase.

Conclusion

Intraoral exposure deteriorated the elastic properties of nickel–titanium (NiTi) archwires but the use of fluorides or antiseptics during orthodontic treatment had no significant effect on the elastic properties of the wires and should have no major clinical implications in changing the orthodontic biomechanics.

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Declarations

Conflict of interest T.Z. Belasic, M. Zigante, M. Uhac, S. Karlovic, I.J. Badovinac and S. Spalj declare that they have no competing interests.

Ethical standards All procedures performed in studies involving human participants or on human tissue were in accordance with the ethical standards of the institutional and/or national research committee and with the 1975 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study. The local ethics committees approved the research, with written informed consent obtained from all subjects or their parents/legally authorized representatives in children under the age of 18 years (Ethics Committee of the Clinical Hospital Centre Rijeka No. 2170-29-02/1-14-2 and Ethics Committee of the University of Rijeka, Faculty of Medicine No. 2170-24-01-14- 05). Trial registration: ClinicalTrials.gov Identifier: NCT03334461.

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