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Impact of periodontal microRNAs associated with alveolar bone remodeling during orthodontic tooth movement: a randomized clinical trial

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Abstract

Background Micro-RNAs (miRNAs) have been reported to play an important role during orthodontic tooth movement (OTM) through the regulation of periodontal soft and hard tissue homeostasis and functions. The aim of the present study was to assess the effects of miRNAs on OTM and to evaluate possible predictors that influenced the overall OTM amount at a 3-month follow-up.

Methods Through a split-mouth design, 21 healthy patients (mean age 13.2 ± 1.8 years) were enrolled in the present study. Clinical parameters and gingival crevicular fluid (GCF) sampling were performed on both compression and tension sides of a random canine to be distalized (test groups) at baseline and at 1 h, 1 day, 1 month and at 3-month after OTM, while the contralateral canine served as a control group. miRNAs – 7a-3p, -7a-2-3p, -7a-5p, -21-3p, -21-5p, -100-3p, -100-5p, -125b-2-3p, -125b-5p, -200b-3p, and – 200b-5p expression was analyzed using a real-time quantitative polymerase chain reaction (RT-PCR). Data were analyzed to assess miRNAs change following OTM. Spearman test, two-way ANOVA and a multivariate regression model were established to evaluate the correlation among miRNAs and clinical parameters and to explore possible predictors of OTM amount at 3-month follow-up.

Results At 3-month follow-up, there was an increase of miRNA-7a-2-3p, -21-5p, -100-5p, a decrease of miRNA-125b-5p, 200b-3p and – 200b-5p in the compression side and an increase of miRNA-7a-3p, 100-5p in the tension side ($p < 0.05$). The two-way ANOVA revealed that OTM determined, on the compression side, a significant upregulation on miRNA-7a-3p ($p = 0.017$), -7a-2-3p ($p = 0.023$), -21-5p ($p = 0.007$), -100-5p ($p = 0.025$) and a significant downregulation of miRNA-125b-2-3p ($p = 0.019$) and – 200b-5p ($p = 0.017$). The multivariate model highlighted that high baseline miRNA-7a2-3p ($p = 0.025$), -21-5p ($p = 0.014$), -200b-3p ($p = 0.041$), young age ($p = 0.042$), lower bleeding on probing (BOP) ($p = 0.021$) and miRNA-125b-2-3p ($p = 0.021$) levels were significant predictors of OTM at 3-month follow-up.

Conclusions In the present study, OTM significantly impacted the expression of the miRNAs analyzed, in both the tension and compression side of traction tooth at 3-month follow-up. High baseline miRNA-7a2-3p, -21-5p, -200b-3p,

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and lower miRNA-125b-2-3p, together with younger age and lower BOP, were significant predictors of OTM amount at 3-month follow-up.

Trial registration ClinicalTrials.gov NCT06023433 (retrospectively registered).

Keywords Tooth movement, microRNA, Periodontal ligament, Biomarker, Osteoclastogenesis, Clinical trial

Background

Orthodontic tooth movement (OTM) is a dynamic multifactorial process that occurs following the application of continuous orthodontic forces associated with periodontal tissues and alveolar bone remodeling [1, 2]. Immediately after the application of an orthodontic force, there is a release of several inflammatory mediators such as prostaglandins, interleukin-1 (IL-1), cytokines and nitric oxide [3] with the concomitant reduction of the local blood flow and related epigenetic process of bone and tissues which determines tooth movement [4].

In this regard, it has been previously reported that the stress induced by an experimental force determines, in periodontal ligament (PDL) cells, an immediate upregulation of nuclear factor kappa-ligand (RANKL), which consequently stimulates the production of osteoclasts aimed at resorbing the bone in the compression area and promoting the tooth movement [5]. At the same time, on the tension side, the osteoblasts will act as stimulators on several mediators related to PDL osteoid deposition, mineralization, and new alveolar bone apposition [6].

MicroRNAs (miRNAs), a small noncoding RNA, have been reported to induce degradation or translational repression of mRNA with a length of 17–24 nt (nucleotides) and to regulate gene functions at the post-transcriptional level in a variety of biological tissues, including PDL and alveolar bone [7–11]. By controlling the differentiation and function of osteoblasts/osteoclasts, certain miRNAs have been associated with regulating both osteoblastogenesis and osteoclastogenesis in some in vitro models [12–14]. In this regard, some preliminary evidence has shown that certain miRNAs, such as miRNA-34a and −27 are able to influence tooth formation, tooth mobility, and odontogenesis through their effects on target genes and pro-inflammatory mediators such as receptor activators of nuclear kappa ligand (RANKL), osteoprotegerin (OPG) and several cytokines [15–17] and also to modulates the production of human PDL stem cells immediately following mechanical stimulation [17, 18]. Furthermore, miRNAs have been shown to play an important role in the regulation of bone remodelling through the epigenetic process of the activation/inhibition of osteoblasts and osteoclasts [19]. Specifically, some studies evidenced that miRNAs-21 are sensitive to stimulating, in both in vitro [18] and in vivo models [20, 21], the differentiation of osteoclast due to a direct RANKL activation that could finally influence the OTM amount. In this regard, some in vivo studies revealed that GCF secretory miRNA-29 [22] and miRNA-34 [7] expression profiles increase during bone activity and OTM in humans and that their levels were influenced by some oral health clinical outcomes such as bleeding on probing (BOP) and plaque index (PI) parameters that have been shown to critically impact the gingival crevicular fluid (GCF) concentrations of miRNA-7, -21, -100, and −125b and systemic inflammatory biomarkers in periodontitis patients [23–26].

Considering the evidence regarding the role of miRNAs, investigating a pattern of GCF miRNAs linked with osteoclastogenesis and oxidative stress could be of interest and may offset the biological effects of miRNAs during the early stages of OTM. Therefore, the aim of this study is to assess the impact of a novel pattern of GCF miRNAs during the early stages of OTM following the application of a controlled orthodontic force. Furthermore, it was explored the correlation among miRNAs concentrations and the amount of OTM (set as primary ourcome variable) and the possible predictors that influenced the overall OTM amount at 3-month follow-up.

Materials and methods

Study design

A randomized-controlled split-mouth study was conducted to evaluate the impact of miRNAs during OTM and the features that influenced the OTM amount in a clinical model. The null hypothesis tested if, at 3-months follow-up, there were no differences in miRNAs expressions among analyzed groups as well as no predictors of OTM amount. The study followed the guidelines of the Helsinki Declaration on medical research reviewed in 2013. All enrolled patients or their parents were informed about the study characteristics and risks and signed their consent before their enrollment. The ethical approval was obtained from the local Institutional Review Board (24/12/PAR), and the study protocol was retrospectively registered on ClinicalTrials.gov (NCT06023433), according to the CONsolidated Standards of Reporting Trials (CONSORT) guidelines.

Study sample

For the present study, consecutive patients requiring bilateral maxillary first premolar extractions for orthodontic purposes were assessed for eligibility at the Dental School of the University of Catania, Catania, Italy.

The inclusion criteria were (1) age between 10 and 18 years; (2) high standards of oral hygiene throughout the study period. The exclusion criteria were (1) poor oral hygiene or periodontal diseases (BOP and Plaque Index-PI, >10%); (2) using medications, steroids or other anti-inflammatory drugs; (3) the presence of any systemic diseases; (4) the presence of bruxism or parafunctions or temporomandibular joint disorders. At baseline and each follow-up session, periodontal parameters such as probing pocket depth (PPD) and clinical attachment loss (CAL) were also recorded.

Reliability evaluation and sample size analysis

Two independent calibrated examiners (A.P. and A.L.G.) assessed the clinical outcomes. The inter-examiner reliability was evaluated on 15 randomly selected patients by setting cumulative mm of distalization of the canines as the primary outcome measure [27]. Using the intraclass correlation coefficient (ICC), the reliability yielded an agreement for the primary outcome [ICC=0.819 (95% CI 0.804–0.826), first examiner; ICC=0.821 (95% CI 0.810–0.829), second examiner], indicating a high level of reliability among examiners.

The sample size calculation was obtained by choosing the OTM amount as a primary variable using statistical software (G*POWER; Universität Düsseldorf, Düsseldorf, Germany). By assuming a mean difference of 1 mm (0 mm, control side; 1 mm, experimental side) in the total amount of OTM among groups, a standard deviation (SD) of 0.75 mm, a power of 95%, and 0.05 as alpha error, a sample size of 16 analyzed teeth per group were needed in order to reach a good power sample [28]. However, in order to minimize the effect of potential dropouts, 21 teeth per group were enrolled.

Study outcomes and measures

The primary outcome evaluated the impact of OTM on GCF miRNAs 7a-5p, 21-3p, 21-5p, 100-5p, 125-5p, 200b-3p, and 200b-5p in both tension and compression sides of the test groups versus control groups at 3-month follow-up. The secondary outcomes evaluated the correlation among miRNAs concentrations and the amount of OTM at -1 and −3-months follow-up to determine the impact of miRNA on OTM amount and to analyze possible predictors of the final OTM amount at 3 months follow-up. These specific miRNAs were selected based on previous evidence highlighting their critical role in bone remodeling and the regulation of osteoclastogenesis during orthodontic tooth movement. For instance, miR-21 [29, 30] is known to influence osteoclastic activity through the RANKL/OPG pathway, while miR-7 [31], miR-100 [32] and miR-125 [33] have been linked to the regulation of osteoblast and osteoclast differentiation. Moreover, the miR-200 family was linked to periodontal inflammation, but no specific data on OTM were available [23].

Randomization

Patients were randomized to a test or control group by an operator not involved in the clinical trial, generating a random computer allocation sequence in a 1:1. Concealment was achieved by assigning subjects to each group by use of sealed and numbered envelopes. Details of the assignment were not shared with the clinicians who performed the treatment or examination.

Treatment and GCF collection

Each individual underwent an oral objective and amnestic evaluation during the initial visit.

After 2 months from the extraction of 2 upper first maxillary premolars, straight wire fixed Roth appliances with 0.022×0.028-inch slots (Mini 2000; Ormco, USA) were applied in each patient by the same operator. Brackets with vertical slots were bonded on canines. Leveling and alignment were then started and considered complete when a 0.017×0.025-inch stainless steel archwire could be placed passively in all the maxillary teeth [34]. Before the onset of canine retraction, the maxillary second premolars and first molars were ligated together on the experimental side using a 0.010-inch wire in the figure-of-eight. The maxillary incisors and the posterior segments were ligated together to aid in stabilization and to prevent their potential spacing.

On the test side, the canine retraction was started by sliding mechanics on 0.017×0.025-inch stainless steel archwire using a nickel-titanium (NiTi) closed coil spring (0.9 mm × 12 mm, Ormco, USA), extended between the first molar hook to the canine bracket's power arm (8 mm in vertical length from the horizontal slot) delivering a force of 150 g approximately, as measured by a force gauge (Morelli, Brazil) [34, 35]. The choice of 0.017×0.025-inch stainless steel archwires for canine retraction was made to provide sufficient rigidity and control of tooth movement. This configuration minimized unwanted tipping or rotation of the canine during distalization [36, 37]. Moreover, the NiTi springs were selected due to their well-documented superelastic properties, which allow for the delivery of a relatively constant force over an extended range of activation. This characteristic is particularly advantageous for maintaining consistent force levels during tooth movement, reducing the need for frequent adjustments, and ensuring a stable application of force throughout the study period. The force magnitude of 150 g was chosen based on existing literature, which suggested that forces in the range of 150–200 g are effective for inducing controlled tooth movement [38–40]. In this way, the combination of

a NiTi coil spring and stainless steel archwire provided a balance between force application and control.

The contralateral canine was considered as a control, and no OTM was applied until the end of the experiment. Then, the control side underwent the same treatment.

The GCF was collected by the same calibrated operator between 9:00–11:00 a.m., at baseline (before applying OTM), and at 1 h, 1 day, 1 week, 1 month and at 3 months after OTM on the distal (compression side) and mesial (tension side) of the test canine to be distalized and on the contralateral canine (control group). Each patient was asked not to eat, drink, or brush their teeth 6 h before GCF sampling. For the GCF collection, the selected tooth was air-dried and isolated with cotton rollers after the supragingival plaque and saliva around them were gently removed with a cotton pellet and two sterile paper strips (Periopaper, Oraflow, NY, USA) were gently inserted into the gingival sulcus for 30 s, as previously described [23]. The paper strips containing blood were discarded.

miRNAs analysis

For the miRNAs analysis, the GCF strips were placed into sterile microtube vials and kept at -80 °C until analysis. According to the manufacturer's instructions, small RNA was extracted from GCF using a kit [miRNeasy® Mini Kit (Qiagen, #217004)] and kept at -80 °C. The sample volume was calculated based on the levels of absorption of the gathered strips, taking into account the 1.2 µl of GCF that is entirely absorbed by a single strip. In order to load the same amount of nucleic acid into each experiment, miRNAs were isolated from each sample. Additionally, as previously mentioned [23], miRNAs were standardized to a reference miRNA U6. A NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Milan, Italy) was used to measure the concentration of RNA and analyze its quality. The GCF volume was quantified using an electronic device (Periotron 8000, Oraflow, NY, USA) and by reporting results in picograms per microliter (pg/ml).

miRNA quantification by reverse-transcriptase PCR (RT-PCR)

The TRIzol kit was used to perform total RNA extraction (Life Technologies, Gaithersburg, MD). The miRCURY LNA RT Kit (catalogue no. 339340) was used to reverse-transcribe 10ng of RNA into complementary DNA (cDNA). Using a 3 µl diluted 1:60 cDNA template of RT-product, PCR processes were performed on a QuantStudioTM 3 (Applied Biosystems Thermo Fisher Scientific, Milan, Italy). Three different experiments have each used three copies of the reactions. The triple mean has been used to handle triplicate results for real-time PCR. The miRCURY LNA SYBR® Green PCR kit (cat. N° 339346) has been used to measure the expression levels of the following miRNAs: miR-7a-3p, miR-7a-2-3p, miR-7a-5p, miR-21-3p, miR-21-5p, miR-100-3p, miR-100-5p, miR-125b-2-3p, miR-125b-5p, miR-200b-3p, miR-200b-5p. After normalization with a reference control, the relative miRNA expression levels have been assessed using the $\triangle\triangle$Ct technique. According to earlier descriptions, the reference gene has been U6 snRNA [23].

Statistical analysis

The clinical data and miRNAs expression (numerical variable) were synthesized as median and interquartile range, while categorical variables were described as numbers and percentages. A non-parametric approach was used since most of the variables were not normally distributed, as verified by the Kolmogorov-Smirnov test. The comparison between groups was performed using the Mann-Whitney test for numerical parameters. The test unit chosen was the single tooth. For both groups, the Friedman test was applied to perform a comparison within each paired group. The numerical variables were compared at baseline and at the different follow-up sessions (baseline, 1 h, 1 day, 1 week, 1 month, and 3-months). To obtain an adjusted P-value for multiple comparisons, the Bonferroni correction was applied (as per protocol analysis for the multiple comparisons), and the α-level of 0.050 was divided by the number of possible intragroup comparisons ($n=15$) among follow-up sessions so that the adjusted intragroup significance level was set at <0.003. The two-by-two comparisons between dependent groups were performed using the Wilcoxon test.

The Spearman's correlation test was used to examine potential correlations between the amount of OTM and the analyzed miRNAs in both tension and compression sides in the analyzed teeth at 1 month and at 3 months following OTM.

Following a logarithmic transformation of miR-7a-3p, miR-7a-2-3p, miR-7a-5p, miR-21-3p, miR-21-5p, miR-100-3p, miR-100-5p, miR-125b-2-3p, miR-125b-5p, miR-200b-3p, miR-200b-5p and obtaining the normality condition after transformation, a two-way ANOVA was used to analyze the impact of OTM on each analyzed miRNA (as continuous variables) changes and to estimate whether these variables changed based on two categorical variables such as amount of OTM (I) and timing of OTM (II). It was evaluated how OTM, alone and in combination, influenced miRNAs changes in tension and compression test groups (OTM was set as a reference).

Finally, uni-and multivariable linear regression models were estimated to assess the dependence of the amount of OTM (difference between baseline and 3 months, as dependent variable) from possible predictor variables (independent variables) such as age, sex, BMI, and baseline BOP, PI, PPD, CAL and miRNAs concentrations. For the models, it was firstly estimated all univariate models

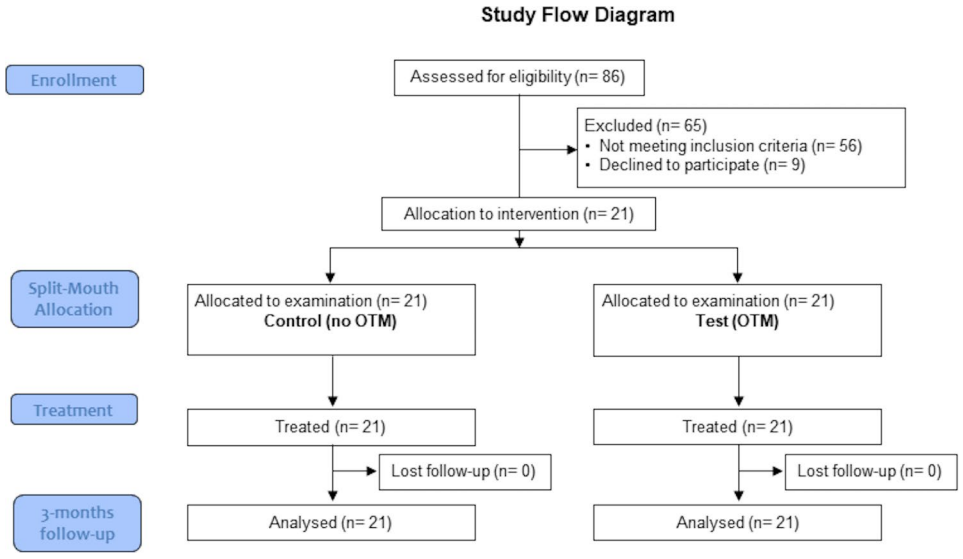

Fig. 1 Workflow of the study

Table 1 Baseline characteristics of the study participants

Variable	Patients ($n=21$)
Age (years), mean ± SD	13.2 ± 1.8
Gender (M/F), no.	10/11
BMI (Kg/m^2), mean ± SD	20.5 ± 2.1
Plaque Index (PI, %), mean ± SD	4.4 ± 1.2
Bleeding on Probing (BoP, %), mean ± SD	7.8 ± 1.0
Probing Depth (PD, mm), mean ± SD	2.47 ± 0.3
Clinical Attachment Loss (CAL, mm), mean ± SD	2.25 ± 0.4

and then it was estimated the multivariate model only the variables that were found to be significant in the univariate approach. Statistical analyses were performed using statistical software (SPSS 22.0; IBM, Bologna, Italy). A p-value lower than 0.05 was considered statistically significant.

Results

Study sample

After the enrollment stage, 65 patients were excluded because they did not meet the inclusion criteria ($n=56$) or declined to participate in the study ($n=9$). Finally, 21 patients were included in the study (10 males, 11 females, mean age 13.2±1.8 years) (Fig. 1). The baseline characteristics of the study participants are presented in Table 1. At baseline, there were no significant differences among control and tests (compression and tension side) groups for PI ($p=0.258$) and BoP ($p=0.336$), PPD ($p=0.445$) and CAL ($p=0.651$).

Primary outcome

On the test side, the mean rate of OTM was 0.94±0.29 (SD) mm at 1 month and 1.36±0.35 (SD) mm at 3 months after OTM.

MiRNAs expression on both the compression and tension sides of the test group and the control group are represented in Table 2. At baseline, there were no differences among groups regarding all miRNA's expression among groups ($p>0.05$). In comparison with control group, in the compression side, at 1 h, OTM determined a significant increase of miRNA-7a-3p ($p<0.001$, compression and tension side), -7a-2-3p ($p<0.05$, tension side), 100-3p ($p<0.05$, tension side) and a significant decrease of miRNA-125b-2-3p ($p<0.001$, tension side), -125b-5p ($p<0.05$, tension side), miRNA-200b-3p ($p<0.05$, compression side), and 200b-5p ($p<0.05$, tension side); at 1 day, there was a significant increase of miRNA-7a-3p ($p<0.001$ compression side, $p<0.05$ tension side), -7a-2-3p ($p<0.001$ compression side, $p<0.05$ tension side), -7a-5p ($p<0.001$, tension side), -21-5p ($p<0.05$) and −100-5p ($p<0.05$, compression and tension side), and a significant decrease of -125b-2-3p ($p<0.001$, compression and tension side), -125b-5p ($p<0.05$, compression side; $p<0.001$, tension side), -200b-3p and −200b-5p ($p<0.001$, tension side); at 1 week, there was a significant increase of miRNA-7a-3p ($p<0.001$, compression and tension side), -7a-5p ($p<0.05$), -21-3p ($p<0.05$, compression side), -21-5p ($p<0.05$, tension side), -100-3p ($p<0.05$, tension side), -100-5p ($p<0.001$, compression side; $p<0.05$, tension side) and a significant decrease of miRNA-125b-2-3p ($p<0.001$, compression side and tension side), -200b-3p ($p<0.001$, tension side) and −200b-5p ($p<0.001$, compression and tension side); at 1 month, there was a significant increase of miRNA-7a-3p ($p<0.001$, compression and tension side), -7a-2-3p ($p<0.05$, compression side) and −21-3p ($p<0.05$, compression side), -21-5p ($p<0.001$ compression side, $p<0.05$ tension side), -100-3p ($p<0.05$, tension

Table 2 miRNAs expression changes at baseline and at each follow-up session. Values are represented such as median and interquartile range (IQR). A, significance between baseline and 1-hour; b, significance between baseline and 1 day; c, significance between baseline and 1 week; d, significance between baseline and 1 month; e, significance between baseline and 3 months; f, significance between 1 h and 1 day; g, significance between 1 h and 1 week; h, significance between 1 h and 1 month; i, significance between 1 h and 3 months; j, significance between 1 day and 1 week; k, significance between 1 day and 1 month; l, significance between 1 day and 1 month; m, significance between 1 day and and 3 months; n, significance between 1 week and 1 month; o, significance between 1 week and 3 months; p, significance between 1 month and 3 months. Data analyzed using the Friedman Test and Dunn Post Hoc analysis. *, $p > 0.05$ versus healthy patients; **, $p < 0.001$ versus healthy patients; †, $p > 0.05$ versus compression side; ††, $p < 0.001$ versus compression side

Time points	Control ($n = 21$)	Test groups ($n = 21$)	
		Compression side	Tension side
miRNA 7a-3p			
Baseline	0.422 (0.2–0.5)	0.429 (0.3–0.5)	0.435 (0.3–0.5)
1 h	0.427 (0.3–0.5)	1.859 (1.6-2) [**]	1.224 (1.1–1.4) [**,†]
1 day	0.431 (0.2–0.5)	2.226 (1.8–2.3) [b, f, **]	1.874 (1.5–1.9) [**,†]
1 week	0.428 (0.2–0.5)	1.751 (1.5–1.9) [c, j, **]	1.236 (1-1.5) [**,†]
1 month	0.415 (0.3–0.5)	1.429 (1.2–1.6) [d, k, **]	0.954 (0.8–1.1) [**,†]
3 months	0.424 (0.3–0.5)	0.685 (0.4–0.7) [**]	0.532 (0.4–0.6) [e, m,o, p]
P-value	0.257	0.048	0.024
miRNA 7a-2-3p			
Baseline	0.669 (0.4–0.7)	0.662 (0.5–0.7)	0.655 (0.5–0.7)
1 h	0.674 (0.5–0.7)	0.996 (0.8–1.1) [a, *]	0.741 (0.6–0.9)
1 day	0.688 (0.5–0.8)	1.112 (1-1.3) [b, **]	1.052 (0.8–1.1) [*]
1 week	0.701 (0.5–0.8)	0.884 (0.8–1.1) [c]	0.714 (0.6-1)
1 month	0.654 (0.4–0.7)	0.859 (0.7–0.9) [d, *]	0.624 (0.5–0.7) [†]
3 months	0.673 (0.5–0.7)	0.902 (0.8-1) [e, *]	0.784 (0.6–0.9) [†]
P-value	0.625	0.012	0.048
miRNA 7a-5p			
Baseline	0.954 (0.8–1.1)	0.926 (0.8–1.1)	0.912 (0.8-1)
1 h	0.941 (0.7–1.1)	1.025 (0.8–1.1) [a]	1.125 (0.9–1.1)
1 day	0.885 (0.7-1)	0.995 (0.7–1.1)	1.326 (1.1–1.4) [b, **, ††]
1 week	0.901 (0.8-1)	1.325 (1.1–1.4) [c, *]	1.442 (1.2–1.6) [c, g, **]
1 month	0.906 (0.7–1.1)	0.952 (0.7-1) [n]	0.995 (0.8–1.1) [l]
3 months	0.922 (0.8-1)	0.901 (0.8–1.1) [o]	0.974 (0.8–1.1) [m]
P-value	0.061	0.028	0.036
miRNA 21-3p			
Baseline	0.774 (0.6–0.8)	0.802 (0.7–0.9)	0.786 (0.6–0.9)
1 h	0.785 (0.6–0.9)	0.912 (0.6–1.2)	0.915 (0.8–1.1)
1 day	0.802 (0.7-1)	1.023 (0.8–1.2)	0.996 (0.8–1.1) [b]
1 week	0.845 (0.6–0.9)	1.335 (1.1–1.4) [c, *]	0.941 (0.7–1.2) [c, †]
1 month	0.885 (0.7–0.9)	1.299 (1.1–1.4) [d, *]	0.915 (0.8-1)
3 months	0.789 (0.6–0.9)	0.956 (0.7–1.1)	0.902 (0.7–1.2)
P-value	0.102	0.044	0.021
miRNA 21-5p			
Baseline	1.112 (0.8–1.3)	1.114 (1-1.4)	1.052 (0.8–1.1)
1 h	1.147 (1-1.2)	1.352 (0.9–1.5) [a]	1.252 (1-1.3)
1 day	1.198 (1-1.3)	1.396 (1.1–1.5) [b]	1.289 (0.9–1.2) [b, *]
1 week	1.136 (0.9–1.2)	1.784 (1.1–1.9) [c, g]	1.432 (1.1–1.5) [*]
1 month	1.087 (0.8–1.2)	1.885 (1.5–2.1) [d, h, **]	1.236 (1-1.4) [*]
3 months	1.095 (0.9–1.2)	1.452 (1.1–1.6) [e, **]	1.209 (1.1–1.4)
P-value	0.247	0.024	0.047
miRNA 100-3p			
Baseline	0.775 (0.7–0.9)	0.789 (0.6–1.5)	0.806 (0.7–0.9)
1 h	0.784 (0.6–0.9)	0.905 (0.7–1.3)[a]	1.112 (0.8–1.1) [*]
1 day	0.795 (0.6–0.9)	0.805 (0.7–1.1)	0.908 (0.7–1.1)
1 week	0.782 (0.5–0.8)	0.841 (0.7–1.1)[c, d]	1.025 (0.8–1.3) [*]

Table 2 (continued)

Time points	Control ($n=21$)	Test groups ($n=21$)	
		Compression side	Tension side
1 month	0.788 (0.6–0.9)	0.806 (0.6–0.9)	1.187 (0.9–1.1) [*, †]
3 months	0.804 (0.6–0.9)	0.829 (0.7–1.1)	1.208 (0.9–1.4) [e, **, ††]
P-value	0.612	0.126	0.039
miRNA 100-5p			
Baseline	0.851 (0.7–0.9)	0.898 (0.7–1.5)	0.906 (0.8–1.1)
1 h	0.882 (0.7–0.9)	1.025 (0.7–1.2)	1.114 (1-1.4)
1 day	0.896 (0.7-1)	1.212 (1.1–1.4) [b, *]	1.296 (1.1–1.4) [*]
1 week	0.884 (0.6–0.9)	1.289 (1.1–1.6) [c, d, *]	1.396 (0.9–1.2) [c, **]
1 month	0.841 (0.7-1)	1.505 (1.3–1.7) [d, h, **]	1.602 (1.2–1.8) [d, h, **]
3 months	0.895 (0.6–0.9)	1.369 (1-1.4) [e, **]	1.424 (1.1–1.6) [e, i, **]
P-value	0.334	0.024	0.041
miRNA 125b-2-3p			
Baseline	1.115 (0.9–1.2)	1.028 (1-1.4)	1.097 (0.9–1.2)
1 h	1.122 (1-1.2)	0.996 (0.8–1.3)	0.556 (0.4–0.6) [a, **]
1 day	1.254 (0.9–1.2)	0.756 (0.9–1.2) [b, **]	0.521 (0.3–0.7) [b, **]
1 week	1.256 (1-1.3)	0.824 (0.7–1.3) [**]	0.606 (0.5–0.7) [c, **]
1 month	1.178 (0.9–1.3)	0.796 (0.5-1) [d, **]	0.687 (0.5–0.8) [d, **]
3 months	1.162 (0.8–1.3)	0.871 (0.6–1.1) [*]	0.957 (0.7–1.1) [h]
P-value	0.128	0.043	0.009
miRNA 125b-5p			
Baseline	0.974 (0.8–1.2)	0.995 (0.8–1.3)	0.936 (0.7)
1 h	0.902 (0.8–1.3)	0.754 (0.6–1.2)	0.601 (0.5–0.9) [a, *]
1 day	0.924 (0.8–1.2)	0.696 (0.5–1.1) [b, *]	0.524 (0.4–0.7) [b, **]
1 week	0.906 (0.8–1.1)	0.702 (0.6–0.8) [c]	0.556 (0.4–0.6) [c]
1 month	0.899 (0.7–1.2)	0.714 (0.6–0.8)	0.665 (0.3–0.8) [d, *]
3 months	0.896 (0.7-1)	0.821 (0.6–0.9)	0.674 (0.4–0.7) [e, *]
P-value	0.147	0.055	0.043
miRNA 200b-3p			
Baseline	1.052 (0.8–1.2)	1.024 (0.9–1.2)	1.097 (0.7–1.2)
1 h	1.085 (0.9–1.1)	0.854 (0.7–1.1) [*]	0.665 (0.4–0.8) [a]
1 day	0.965 (0.8–1.2)	0.884 (0.7–1.3)	0.521 (0.4–0.7) [b, **, †]
1 week	0.994 (0.7–1.3)	0.799 (0.5–0.9) [c]	0.505 (0.3–0.8) [c, **, †]
1 month	0.921 (0.7-1)	0.788 (0.6–0.9) [d]	0.602 (0.5–0.7) [d]
3 months	0.965 (0.8–1.1)	0.905 (0.7–1.2)	0.701 (0.6–0.9) [e, *, †]
P-value	0.159	0.078	0.033
miRNA 200b-5p			
Baseline	0.801 (0.7-1)	0.854 (0.7–1.2)	0.888 (0.7–1.1)
1 h	0.785 (0.6–0.9)	0.741 (0.6–1.2)	0.502 (0.4–0.7) [a, *, †]
1 day	0.796 (0.6–0.9)	0.502 (0.4–0.6) [b]	0.356 (0.2–0.5) [b, **, ††]
1 week	0.812 (0.7-1)	0.489 (0.3–0.7) [c, **]	0.301 (0.2–0.4) [c, **, ††]
1 month	0.801 (0.6–0.9)	0.699 (0.4–0.8) [d, *]	0.412 (0.3–0.7) [d, **, ††]
3 months	0.791 (0.6–0.9)	0.715 (0.5–0.9)	0.526 (0.4–0.8) [e, *, †]
P-value	0.885	0.019	0.008

side), and −100-5p ($p<0.001$, compression and tension side), and a significant decrease of -125b-2-3p ($p<0.001$, compression and tension side), -125b-5p ($p<0.05$, tension side) and −200b-5p ($p<0.05$ compression side, $p<0.001$ tension side); at 3 months, there was a significant increase of miRNA-7a-3p ($p<0.001$, compression side), -100-5p, -21-5p ($p<0.001$, compression side), -100-3p ($p<0.001$, compression side), -100-5p ($p<0.001$, compression and tension side) and a significant decrease of -125b-2-3p ($p<0.05$, compression side), -125b-3p, -125b-5p, 200b-3p, and −200b-5p ($p<0.05$, tension side). Moreover, in comparison with the compression side, in the tension side, at 1 h after OTM, there was a significant decrease of miRNA-7a-3p ($p<0.001$), and −200b-5p ($p<0.05$); at 1 day after OTM, there was a significant decrease of miRNA-7a-3p ($p<0.05$) -7a-5p ($p<0.001$),

miRNA-200b-3p ($p<0.05$) and −200b-5p ($p<0.001$); at 1 week after OTM, there was a significant decrease of miRNA-7a-3p ($p<0.05$), -21-3p ($p<0.05$), -200b-3p ($p<0.05$) and −200b-5p ($p<0.001$); at 1 month after OTM, there was a significant decrease of miRNA-7a-3p, -7a-2-3p ($p<0.05$), and −200b-5p ($p<0.001$) a significant increase of miRNA-100-3p ($p<0.05$); at 3 months after OTM, there was a significant decrease of miRNA-7a-3p, -7a-2-3p ($p<0.05$), -200b-3p and −200b-5p ($p<0.05$) a significant increase of miRNA-100-3p ($p<0.001$).The correlation analysis (Table 3) among the amount of OTM and miRNAs variables evidenced that, at 1 month follow-up, there was a significant correlation among the amount of OTM and miRNA-7a-2-3p (rs=0.247; $p=0.048$), miRNA-21-3p (rs=0.109; $p=0.044$), miRNA-21-5p (rs=0.359, $p=0.005$). The same correlation analysis at 3 months after OTM evidenced that there was a significant correlation among amount of OTM and low BOP (rs=-0.257: $p=0.035$), miRNAs 7a-2-3p (rs=0.187, $p=0.031$), -21-5p (rs=0.331, $p=0.018$), -100-5p (rs=0.197, $p=0.027$), -125b-2-3p (rs= -0.256, $p=0.002$), and −200b-5p (rs=0.445, $p=0.004$).

The two-way ANOVA estimation models established to determine, in the test groups, the impact of OTM on miRNAs at 3 months follow-up revealed that OTM determined, on the compression side, a significant effect on the increase of miRNA-7a-3p ($p=0.017$), -7a-2-3p ($p=0.023$), -21-5p ($p=0.007$), -100-5p ($p=0.025$) and the decrease of miRNA-125b-2-3p ($p=0.019$) and −200b-5p ($p=0.017$). Moreover, the timing of OTM significantly impacted miRNA-7a-3p ($p=0.022$), -21-5p ($p=0.012$), -125b-2-3p ($p=0.019$) and −200b-5p ($p=0.023$) changes at 3 months after treatment (Table 4). Interestingly, the analysis evidenced that patients with high baseline levels of miRNA-7a-3p, -7a-2-3p, -21-5p, -100-5p and lower levels of miRNA-125b-2-3p and −200b-5p gained more OTM amount at 3-months follow-up.

The multivariate regression models, aimed at identifying the baseline impact of possible predictors of OTM amount highlighted that, in all patients, high miRNA-7a2-3p ($p=0.025$), -21-5p ($p=0.014$), -200b-3p ($p=0.041$), young age ($p=0.042$), lower BOP ($p=0.021$) and miRNA-125b-2-3p ($p=0.021$) levels were significant positive predictors of OTM at 3-month follow-up after treatment (Table 5).

Table 3 Correlation analysis at 1- and at 3 months follow up among OTM amount and clinical variables

Variable	1 months OTM		3 months OTM	
	Rs coeff.	*p*-value	Rs coeff.	*p*-value
Age	0.258	0.058	0.114	0.331
Gender	0.104	0.345	-0.214	0.087
BOP	0.425	0.038	-0.257	0.035
Plaque index	0.331	0.052	0.066	0.059
PPD	0.212	0.344	0.145	0.213
CAL	0.156	0.243	0.112	0.556
SES	0.449	0.114	-0.114	0.068
BMI	0.189	0.108	-0.219	0.578
miRNA 7a-3p	0.108	0.587	-0.366	0.147
miRNA 7a-2-3p	0.247	0.048	0.187	0.031
miRNA 7a-5p	0.215	0.087	0.224	0.114
miRNA 21-3p	0.109	0.044	0.239	0.475
miRNA 21-5p	0.359	0.005	0.331	0.018
miRNA 100-3p	0.441	0.067	0.241	0.056
miRNA 100-5p	0.336	0.012	0.197	0.027
miRNA 125b-2-3p	-0.344	0.039	-0.256	0.002
miRNA 125b-5p	-0.386	0.205	0.241	0.257
miRNA 200b-3p	0.257	0.021	0.445	0.004
miRNA 200b-5p	0.442	0.542	0.204	0.478

Discussion

The main objective of this study was to assess the impact of GCF miRNAs related to bone remodelling and oxidative stress on both the tension and compression side during OTM at 3-month follow-up. Moreover, the study evaluated the correlation among miRNAs and OTM amount and was aimed at determining possible predictors of OTM at 3 months follow-up.

In the test group's tension and compression side, the OTM determined the dysregulations of GCF miRNAs. In this regard, the role of miRNAs regulation in oral tissue remodeling and bone homeostasis is already well known beyond orthodontic treatment [41]. OTM is a mechanism that impacts the overall periodontium through physiological alveolar bone adaptation to mechanical stresses. Such movement is accomplished under physiological settings by highly coordinated and effective bone remodeling, which necessitates pairing of bone production and resorption through mechanisms involving inflammatory mediators such as prostaglandins and cytokines and oxidative stress mediators [42, 43]. The result is differentiated cellular activities on the compression side aimed at promoting bone resorption, through a reduced oxygen flow that promotes cell hyalinization, which undermines osteoclastic bone absorption. On the traction side, blood flow is activated, and osteoblasts are activated in osteoid deposition followed by tardive mineralization [44], with the concomitant compression and tension sides which differently stimulates mediators linked with both osteoclasts and osteoblasts activities (e.g. miR-3198) [42].

Interestingly, for all the miRNAs investigated, there was a significant dysregulation of GCF miRNAs levels for both the compression and traction sides already at 1 h and 1 day after OTM. These results are in accordance with previous reports [22] and may be explained for the hyalinization phase (after 24–48 h the application of an orthodontic force) which is characterized by a transient

Table 4 Results of two-way ANOVA for the dependent variable miRNAs at 3 months follow-up. For group, tension served as a reference. MS: Mean of Square. F: Fisher test; Group*Timing: Interaction term. Tension side was set as a reference for group

	miRNA 7a-3p		
Source of variation	*MS*	*F*	*p-value*
Group	235.84	207.35	0.017
OTM Timing	237.28	166.39	0.022
Group*Timing	4.21	5.31	0.036
Within	2.47		
	miRNA 7a-2-3p		
Source of variation	*MS*	*F*	*p-value*
Group	219.23	224.18	0.023
Timing	191.46	235.19	0.084
Group*Timing	3.25	3.58	0.044
Within	2.14		
	miRNA 7a-5p		
Source of variation	*MS*	*F*	*p-value*
Group	221.11	187.12	0.116
Timing	148.15	244.23	0.552
Group*Timing	3.29	3.12	0.302
Within	2.01		
	miRNA 21-3p		
Source of variation	*MS*	*F*	*p-value*
Group	225.15	202.68	0.204
Timing	192.36	251.24	0.314
Group*Timing	3.79	3.74	0.208
Within	2.87		
	miRNA 21-5p		
Source of variation	*MS*	*F*	*p-value*
Group	242.23	314.15	0.007
Timing	204.28	266.29	0.012
Group*Timing	3.89	4.12	0.025
Within	2.85		
	miRNA 100-3p		
Source of variation	*MS*	*F*	*p-value*
Group	212.19	218.96	0.274
Timing	191.25	265.25	0.335
Group*Timing	3.78	2.19	0.875
Within	2.56		
	miRNA 100-5p		
Source of variation	*MS*	*F*	*p-value*
Group	236.19	199.24	0.245
Timing	195.14	246.29	0.332
Group*Timing	3.24	3.57	0.207
Within	2.06		
	miRNA 125b-2-3p		
Source of variation	*MS*	*F*	*p-value*
Group	-206.19	-236.18	0.006
Timing	205.87	225.14	0.019
Group*Timing	3.41	3.78	0.002
Within	2.47		
	miRNA 125b-5p		
Source of variation	*MS*	*F*	*p-value*
Group	155.26	178.26	0.104
Timing	202.25	241.06	0.205
Group*Timing	3.78	2.45	0.441

Table 4 (continued)

	miRNA 7a-3p		
Within	2.22		
	miRNA 200b-3p		
Source of variation	*MS*	*F*	*p-value*
Group	225.19	239.25	0.116
Timing	204.28	241.23	0.547
Group*Timing	3.48	3.05	0.241
Within	2.47		
	miRNA 200b-5p		
Source of variation	*MS*	*F*	*p-value*
Group	228.15	252.15	0.017
Timing	205.21	304.12	0.023
Group*Timing	3.74	3.28	0.006
Within	2.55		

Table 5 Uni- and multivariable linear regression analysis for OTM amount at 3 months follow-up. Significance was set as $p < 0.05$

	Amount of OTM			
	Univariate		**Multivariate**	
Variable	**B**	***p*-value**	**B**	***p*-value**
Age	-0.305	0.025	-0.204	0.042
Gender	0.145	0.236	-	-
BOP	-0.178	0.019	-0.305	0.021
Plaque index	0.102	0.305	-	-
PPD	0.056	0.211	-	-
CAL	0.278	0.432	-	-
SES	-0.202	0.441	-	-
BMI	-0.105	0.206	-	-
miRNA 7a-3p	0.268	0.105	-	-
miRNA 7a-2-3p	0.104	0.012	0.254	0.025
miRNA 7a-5p	0.374	0.298	-	-
miRNA 21-3p	0.205	0.055	-	-
miRNA 21-5p	0.412	0.039	0.202	0.014
miRNA 100-3p	0.205	0.205	-	-
miRNA 100-5p	0.311	0.027	-	-
miRNA 125b-2-3p	-0.241	0.002	-0.228	0.021
miRNA 125b-5p	0.268	0.257	-	-
miRNA 200b-3p	0.357	0.027	0.225	0.041
miRNA 200b-5p	0.257	0.447	-	-

blockage of orthodontic movement and bone remodeling phenomena.

The correlation analysis evidenced that, at 3-months follow-up, there was a significant correlation among the amount of OTM and miRNAs 7a-2-3p, -21-5p, -100-5p, -125b-2-3p, -200b-5p and also with BOP. Moreover, the two-way ANOVA estimation models revealed that OTM and timing of OTM determined a significant effect on the increase of several analyzed miRNAs, evidencing that patients with high baseline levels of miRNA-7a-3p, -7a-2-3p, -21-5p, -100-5p and lower levels of miRNA-125b-2-3p and −200b-5p gained more OTM amount at 3-months follow-up.

In this regard, previous research suggested that miRNAs play a significant role in bone remodeling at a posttranscriptional level by regulating osteoblast/osteoclast activity and differentiation during OTM [45]. For instance, miR-21 supported OTM by controlling the T cell nuclear factor kappa-B ligand/osteoprotegerin (RANKL/OPG) balance [29, 30]. In contrast, the absence of miR-21 inhibited osteoclastogenesis, as suggested by the reduced distance obtained by orthodontic movement [46]. These data are in accordance with our results since a significant increase in GCF miRNAs after the application of active OTM compared to baseline and to the control group. Furthermore, there are studies that demonstrate the involvement of miR-21 in osteogenesis [47–49], suggesting its dual role in promoting bone resorption and apposition in agreement with the present results. In this regard, some previous studies reported down-regulation of miR-21 could induce the up-regulation of target protein PLAP-1 (periodontal ligament associated protein-1) in the late induction of mineralization of PDL cells [50]; however, when loaded stretch forces, upregulated miR-21 promoted osteogenic differentiation of PDL stem cells [18].

Furthermore, the multivariate regression model results, aimed at identifying the baseline impact of possible predictors of OTM amount, highlighted that, in all patients, high miRNA-7a2-3p, -21-5p, -200b-3p, young age, lower BOP and miRNA-125b-2-3p were significant positive predictors of OTM at 3-month follow-up. As for miRNA-21, similar miRNAs (miRNA-100-3-p, -100-5-p, -125b-2-3p, -125b-5p, -200b-3p and −200b-5p) could be involved in osteoclastogenesis and therefore could become targets to enhance this process, having as a clinical result a speeding up of tooth movement. However, our previous study revealed miRNA-200b-3p and −200b-5 involvement in periodontitis, characterized by pathological bone resorption induced by bacterial infection and the host's inflammatory response. More specifically, it

has been observed that miRNA-200b-3p and −200b-5p, biomarkers linked with unpaired oxidative stress status, significantly increased in periodontitis patients [51]. In the present study, these two miRNAs were significantly increased, specifically on the compression side; therefore, they could have a role in bone resorption. According to a recent in vivo study [32], bone resorption and in vitro osteoclast development were inhibited by miR-100-5p overexpression by blocking fibroblast growth factor (FGF) 21, which can over-activate the RANKL/RANK/NFATc1 signaling pathway and osteoclastic bone resorption [32]. Moreover, miRNA-125b has been identified as having a role in inhibiting osteoblastic differentiation of PDL cells through activating NF-κB by interacting with the NF-κB inhibitor interacting RAS-like 2 (NKIRAS2) gene [33]. Finally, miRNA-7 significantly increased the traction side, suggesting their involvement in osteoblastic activity as previously reported through the interaction with some markers of osteoblast differentiation, including alkaline phosphatase, osteocalcin and type I collagen α1 [31].

However, the present study has some limitations, including the relatively small number of patients enrolled and the short follow-up analysis session, a factor which may have better determined the impact of OTM in the short term but was not timing sufficiently to evaluate the long-term influence of miRNAs on OTM. Although the follow-up was limited to three months, the changes in miRNA levels observed may indicate a potential role in the long-term regulation of bone remodeling during orthodontic tooth movement. Future studies should investigate whether altered miRNAs continue to have a significant influence over time, potentially affecting post-treatment stabilization processes and the risk of relapse. Moreover, including patients with higher ages could be useful to better evaluate the impact of age on miRNAs expression during OTM.

Conclusions

The present study results evidenced that OTM significantly impacted the expression of certain CGG miRNAs linked with osteoclastogenesis in the alveolar bone and with oxidative stress in both the tension and compression side at 3-month follow-up after OTM. The same analyzed miRNAs −7, -21, -100, -125 and −200 were significantly correlated with the overall OTM amount at 1- and 3-months follow-ups. Moreover, high baseline miRNAs −7a2-3p, -21-5p, -200b-3p, and lower −125b-2-3p, together with younger age and lower BOP, were significant predictors of OTM at 3-month follow-up. However, further clinical studies with larger samples will be necessary to better define the role of miRNAs in OTM.

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Author contributions
GI and GLV performed the research, contributed to the analysis and wrote the manuscript; AP, ALG performed the analysis, wrote the manuscript; AD and LO performed laboratory analyses, AMA and GP and supervised the research; AA performed the statistical analysis. All the authors read and approved the final manuscript.

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Data availability
All data are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
The ethical approval was obtained from the local Institutional Review Board of the University of Catania, Catania, Italy (24/12/PAR).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interest.

Competing interests
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