



# Unveiling molecular dynamics: the effects of functional mandibular advancement and a one-month recovery period on myostatin and myosin-1c gene expression in masticatory muscles of young male Wistar rats

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## Abstract

**Aim:** Functional mandibular advancement appliances are widely used in orthodontics, but their molecular and histological effects on masticatory muscles remain underexplored. This study evaluated gene expression and tissue-level adaptations in response to mandibular advancement and a subsequent recovery period in young male Wistar rats.

**Materials and methods:** Thirty rats were randomly assigned to three groups: control (Group A), functional mandibular advancement for four weeks (Group B), and functional mandibular advancement followed by a one-month recovery period (Group C). The expression of myostatin and myosin-1c genes was determined using quantitative real-time reverse transcription polymerase chain reaction. Histological analyses were performed to examine muscle fiber morphology in the masseter and lateral pterygoid muscles.

**Results:** Mandibular advancement produced a significant downregulation of myostatin and upregulation of myosin-1c ( $p < 0.05$ ), most prominent in the lateral pterygoid muscle. Histological evaluation revealed hypertrophic changes in muscle fibers consistent with increased mechanical loading. After the recovery phase, altered gene expression and hypertrophic features persisted partially, indicating sustained molecular and structural adaptations. The masseter and lateral pterygoid muscles exhibited distinct, muscle-specific responses at both the genetic and histological levels.

**Conclusion:** Mandibular advancement induces molecular and histological adaptations in masticatory muscles, with partial persistence following a one-month recovery period. These results suggest the potential for long-term functional and morphological changes after orthodontic intervention and provide biological evidence to inform personalized treatment and retention strategies in growing individuals.

## Keywords

functional appliance, gene expression, mandibular advancement, masticatory muscles, myostatin, myosin-1c, young male Wistar rats

## Introduction

Functional appliances are widely used in orthodontic practice to modulate craniofacial growth, particularly during adolescence. These devices apply controlled mechanical forces to the jaws, promoting orthopedic and muscular adaptations. Among the tissues affected, masticatory muscles—which include the masseter and lateral pterygoid muscles—are subject to significant functional remodeling in response to altered mandibular positioning.

This biological response aligns with the Functional Matrix Theory (FMT) proposed by Moss<sup>[1,2]</sup>, which posits that soft tissue functional demands drive the growth and form of craniofacial skeletal structures. According to FMT, organs such as muscles, the tongue, and orofacial soft tissues guide the spatial and temporal development of bones via dynamic mechanical stimuli. This theory has important implications not only in orthodontics but also in craniofacial developmental biology and oral rehabilitation.

From a molecular perspective, gene expressions within masticatory muscles intricately regulate muscle growth, hypertrophy, and remodeling.<sup>[3,4]</sup> Of particular interest is myostatin—a known negative regulator of skeletal muscle growth—which limits hypertrophy and maintains muscle homeostasis.<sup>[5,6]</sup> In contrast, myosin-1C (MYO1C), although less studied in the context of skeletal muscle, is emerging as a potential modulator of intracellular transport, actin dynamics, and adaptive muscular responses to mechanical loading.<sup>[7-9]</sup>

In this study, we focused on two key masticatory muscles: the masseter, essential for jaw closure and force generation during mastication, and the lateral pterygoid, which facilitates mandibular protrusion and jaw opening.<sup>[4]</sup> These muscles are directly engaged by functional appliances, particularly those designed for mandibular advancement. Previous research has primarily concentrated on the condylar response to such appliances<sup>[9-12]</sup>, but few have examined the molecular and structural changes within the muscles themselves.

Understanding their molecular response may shed light on how muscle adapts to altered functional demands, a question relevant to orthodontists, oral surgeons, and craniofacial researchers alike.

Moreover, most studies focus solely on the active phase of treatment, with little consideration given to what happens after the appliance is removed—when tissues may begin to revert or stabilize. Furthermore, the phenomenon of transient acceleration of growth during functional appliance therapy—whereby orthodontic forces stimulate short-term skeletal adaptation—has been widely acknowledged.<sup>[8,9]</sup> This study introduces a novel element: the inclusion of a one-month post-treatment recovery phase, designed to observe whether gene expression changes induced by mandibular advancement persist or normalize over time. We hypothesize that functional mandibular advancement will induce measurable changes in myostatin and MYO1C expression, alongside structural remodeling,

and that some of these changes may persist after treatment cessation.

By bridging the gap between orthodontic mechanics, muscle biology, and molecular genetics, this investigation contributes to a deeper understanding of the adaptability of craniofacial musculature—a topic that transcends specialty boundaries and informs clinical decision-making in multiple dental disciplines.

Furthermore, most studies focus solely on the active phase of treatment, with little consideration given to what happens after the appliance is removed—when tissues may begin to revert or stabilize.

## Materials and methods

### Animal model

The Wistar rat was selected due to its well-characterized genome and physiological similarities to human craniofacial development, making it a reliable model for evaluating muscle and skeletal responses to functional mandibular advancement. The study involved healthy male Wistar rats aged 6–8 weeks, chosen to align with the growth phase relevant for interventions. Female rats and those outside the specified age range were excluded.

All procedures adhered to ethical animal research guidelines, including the ARRIVE guidelines, and were approved by the Institutional Animal Ethics Committee (IAEC/CPC-SEA/013/2022).

### Experimental design

#### Sample size estimation

The required sample size was calculated using the formula for comparing two means:

$$n_1 = \frac{(\sigma_1^2 + \frac{\sigma_2^2}{\kappa}) (Z_{1-\alpha/2} + Z_{1-\beta})^2}{\Delta^2}$$

where:

$\sigma_1$  = standard deviation of Group 1

$\sigma_2$  = standard deviation of Group 2

$\kappa = \frac{n_2}{n_1} = 1$  (ratio of group 2: group 1)

$Z_{(1-\alpha/2)}$  = two-sided Z value (e.g., 1.96 for 95% confidence interval)

$Z_{(1-\beta)}$  = Z value for power (e.g., 0.84 for 80% power)

$\Delta$  = difference in means to detect (effect size)

Thus, the minimum required sample size per group was 10 rats. Considering three experimental groups, the total sample size was set at 30 rats.

Thirty male Wistar rats were randomly assigned to three groups (n=10 per group):

- Group A – control group: no appliance was applied.

- Group B – appliance group: rats received a mandibular advancement appliance for 1 month.
- Group C – appliance + recovery group: rats received the appliance for 1 month, followed by a 1-month recovery period without the appliance.

Each rat was housed individually under standardized conditions—a 12-hour light/dark cycle, temperature of 25°C–30°C, and 60% humidity. Animals were provided standardized pellet diets and ad libitum water access. Monitoring was performed every 3 hours throughout the study to assess feeding behavior and detect any appliance-related irritation. Dislodged appliances were replaced immediately to maintain consistent mandibular advancement.

### Appliance fabrication and functional advancement protocol

Custom mandibular advancement appliances were fabricated using alginate impressions of the lower incisors and self-cure acrylic resin. The appliance incorporated a 4 mm

mandibular advancement consistent with functional orthopedic studies.<sup>[12-14]</sup> The device was designed to induce forward and downward mandibular positioning during both rest and function.

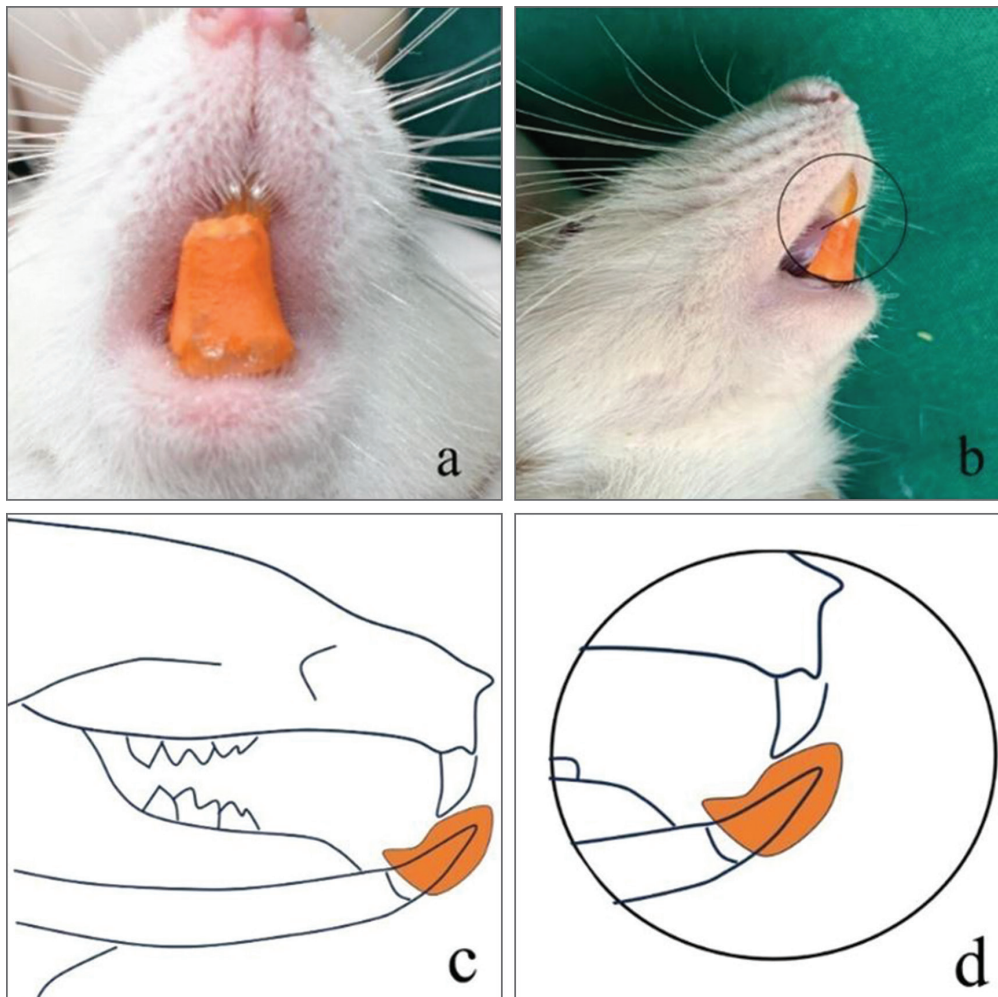
Prior to appliance placement, each unit was checked for fit to avoid mucosal irritation. Under ether sedation (15% w/v), the appliances were cemented to the lower incisors using light-cure resin cement. To prevent occlusal interference, upper incisors were trimmed to control for continuous eruption (**Fig. 1**).

Body weight was tracked as a health indicator throughout the experiment.

### Sample collection

At the end of the first month:

- Groups A and B rats were euthanized via decapitation for tissue harvest.
- Group C rats were euthanized after an additional one-month recovery phase.



**Figure 1.** A mandibular advancement bite-jumping appliance was custom-fabricated specifically for the lower jaw and securely affixed to the lower incisor teeth. **a)** An anterior view presenting the appliance; **b)** A lateral perspective showcasing the inclined plane of the appliance; **c)** A schematic representation illustrating the appliance's structure; **d)** An enlarged schematic portrayal providing a detailed view of the appliance's design.

The following tissues were bilaterally dissected:

- Masseter muscle
- Lateral pterygoid muscle

Tissue from the right side was designated for gene expression analysis, and left-side samples were preserved for histomorphometric evaluation. Muscle samples were weighed and cryopreserved at  $-80^{\circ}\text{C}$  until analysis.

## Gene expression analysis

We analyzed the gene expression of myostatin, a negative regulator of muscle growth, and MYO1C, which is involved in intracellular transport and cytoskeletal regulation. Total RNA was extracted using the standard Trizol protocol. Quantitative real-time RT-PCR was carried out using TaqMan probe-based chemistry on a 96-well plate with the Applied Biosystems QuantStudio™ Real-Time PCR System (v. 1.5.2). Each sample was run in triplicate for technical accuracy. The housekeeping gene  $\beta$ -actin was used for normalization.

Gene expression was quantified using the  $2^{-\Delta\Delta\text{Ct}}$  method, calculated as follows:

1.  $\Delta\text{Ct} = \text{Ct}(\text{target gene}) - \text{Ct}(\beta\text{-actin})$
2.  $\Delta\Delta\text{Ct} = \Delta\text{Ct}(\text{experimental group}) - \Delta\text{Ct}(\text{control group})$
3.  $\text{Fold change} = 2^{-\Delta\Delta\text{Ct}}$ 
  - Fold change  $>1$  = upregulation
  - Fold change  $<1$  = downregulation

Statistical analysis was conducted using SigmaPlot v. 13. Primer and probe sequences are provided in **Table 1**.

## Histological analysis

Muscle biopsies were processed at Excel Diagnostics, Pune. Tissues were fixed in 10% neutral buffered formalin (NBF) for 72 hours and subsequently processed using standard histological protocols, including dehydration in graded alcohols (50%–100%), clearing in xylene, embedding in paraffin, and sectioning into  $5\ \mu\text{m}$  slices using a Leica microtome.<sup>[15,16]</sup> Sections were stained using the Periodic Acid-Schiff (PAS) method and examined under

a Nikon-equipped binocular microscope. All histological evaluations were performed by a blinded veterinary pathologist. The following histomorphometric parameters were assessed: muscle fiber diameter, connective tissue proliferation, and the presence of inflammatory infiltrates. Objective measurements were obtained using ImageJ image analysis software.

## Statistical analysis

Data were expressed as the mean  $\pm$  standard deviation. The assumption of normality was verified using the Shapiro-Wilk test. One-way ANOVA was performed for intergroup comparisons, followed by Tukey's post-hoc test for evaluating pairwise differences. A  $p$ -value  $<0.05$  was considered statistically significant. Where applicable, effect sizes and 95% confidence intervals were also calculated to assess the magnitude and precision of the observed differences. SigmaPlot v. 13 software was used for data analysis.

## Results

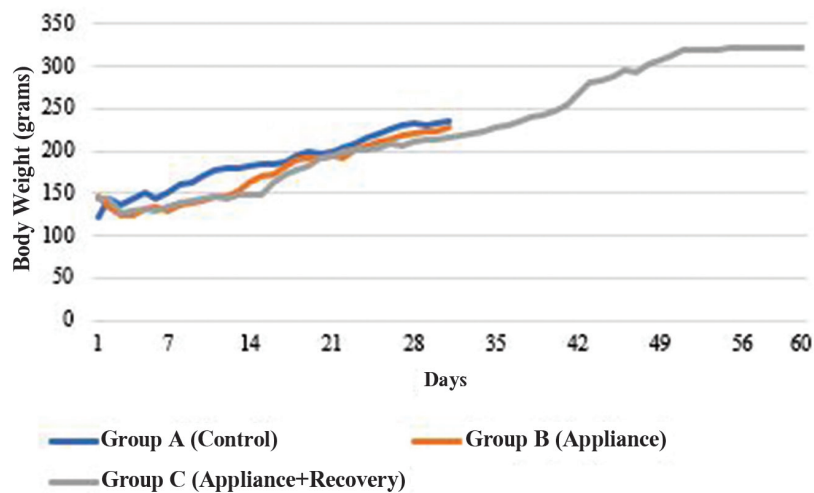
The investigation primarily focused on evaluating gene expression changes in masticatory muscles, aiming to decipher the responsiveness of myostatin and MYO1C to orthodontic interventions.

Rats were weighed and measured daily from the commencement of the study until euthanasia. A slight, statistically insignificant ( $p>0.05$ ) decrease in weight was noted during the initial ten days following appliance placement in the experimental group, attributed to the presence of the appliance. However, weight gradually increased thereafter, reaching levels comparable to the control group as the experimental group acclimated to the mandibular advancement bite-jumping appliance (**Fig. 2**).

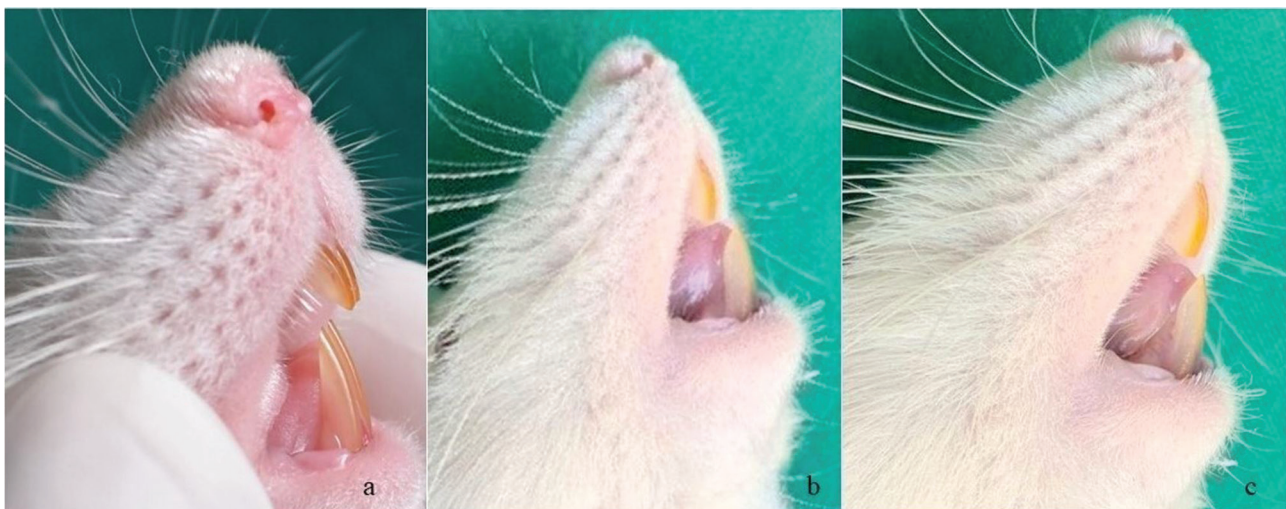
Upon removal of the appliance, rats in the experimental group exhibited an anterior crossbite, as depicted in **Fig. 3b**. This crossbite persisted during the recovery phase

**Table 1.** PCR primers and probes for gene amplification

S.No.	Oligonucleotide name	Primer and probe	Sequence
1	Beta actin (Housekeeping gene)	Forward Primer	TGGATGACGATATCGCTGC
		Reverse Primer	CTTCTGACCCATACCCACCA
		Probe	FAM-CGACAACGGCTCCGGCATGT-TAM
2	MYO1C	Forward Primer	CCTGGTGGAGGAGAAATTCA
		Reverse Primer	GTGTCCTCCAGCTTCTCCAG
		Probe	FAM-CGTCCTGGGGAGGCCACAGA-TAM
3	Myostatin	Forward Primer	AAAGAGGGGCTGTGTAATGCG
		Reverse Primer	TCCGTGGTAGCGTGATAATCG
		Probe	FAM-ACTCCGCCTGGAAACAGCGC-TAM



**Figure 2.** The figure illustrates the average body weight of rats throughout the duration of the experiment. Body weight was recorded at regular intervals in both control and experimental groups. A minor, statistically insignificant reduction was observed in the initial phase, likely due to adjustment to the mandibular advancement appliance.



**Figure 3.** The maxillomandibular incisor relation of the rats was observed under the following conditions: a) prior to appliance insertion; b) following appliance removal; and c) after a one-month recovery period.

but appeared less pronounced compared to the appliance group (Fig. 3c).

## Gene expression in masticatory muscles

### *Myostatin gene expression in masticatory muscles*

#### A) Masseter muscle

The impact of interventions on myostatin gene expression in the masseter muscle was investigated and the following results were observed (Fig. 4, Table 2).

The mean gene expression levels for myostatin levels in masseter muscle were as follows:

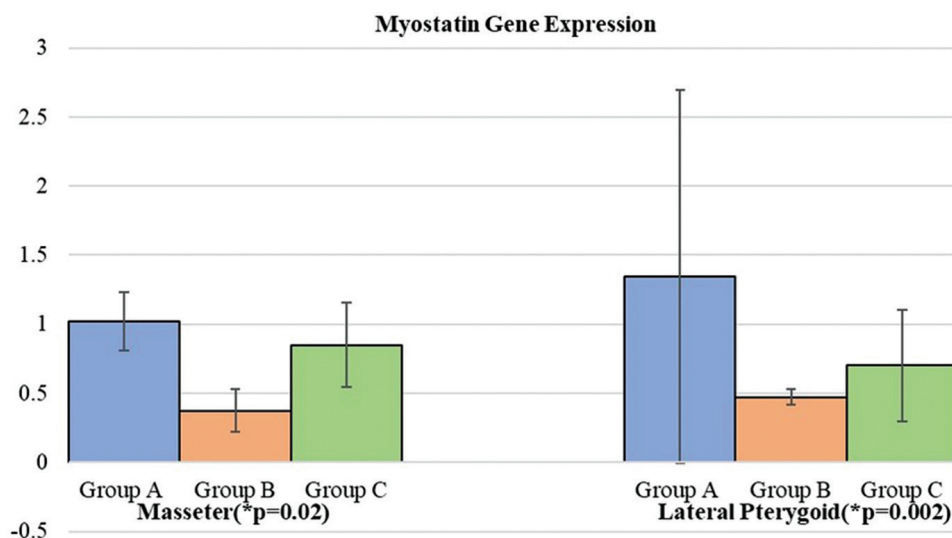
Group A (Control): Mean  $\pm$  SD = 1.01  $\pm$  0.21

Group B (Appliance): Mean  $\pm$  SD = 0.37  $\pm$  0.15

Group C (Appliance + Recovery): Mean  $\pm$  SD = 0.85  $\pm$  0.31  
The ANOVA test indicated a significant difference between the groups ( $p=0.02^*$ ), suggesting that at least one of the groups had a different mean gene expression level compared to the others. Upon applying pairwise comparisons:

- Group A vs. Group B: A significant difference was found in gene expression levels ( $p=0.02940$ ) between these two groups. Group A had a higher mean gene expression compared to Group B.
- Group A vs. Group C: No significant difference was found in gene expression levels ( $p=0.77643$ ) between these two groups.
- Group B vs. Group C: No significant difference was found in gene expression levels ( $p=0.12223$ ) between these two groups.

The myostatin gene expression in the masseter muscle appears to be influenced by the intervention (appliance and



**Figure 4.** Bar graph illustrating myostatin gene expression levels in the masseter and lateral pterygoid muscles. The data, derived from a sample size of 10 for each of the three groups, was analyzed using One-way ANOVA, and significant differences are denoted by \* $p < 0.05$ .

**Table 2.** Mean fold change values of myostatin expression in masseter muscle. One-way ANOVA based on ranks test was done to compare between three groups of masseter muscle for myostatin gene expression (\*the result was significant at  $p < 0.05$ ). Pairwise comparisons with Tukey’s HSD for myostatin gene expression in the masseter muscle, showcasing mean differences, critical values (HSD), and p-values for significant group distinctions (\* $p < 0.05$ ). SD: standard deviation, n: sample size

S.No.	Group A (Control) n=10	Group B (Appliance) n=10	Group C (Appliance+Recovery) n=10
1	1.09	1.35	0.06
2	0.7	0.07	2.89
3	1.16	0.05	0.36
4	1.13	0.03	0.09
5	1.29	0.06	1.1
6	0.85	0.45	0.74
7	0.79	0.15	0.6
8	1.15	0.58	1.03
9	1.23	0.47	1.11
10	0.77	0.49	0.56
<b>Mean±SD</b>	1.01±0.21	0.37±0.15	0.85±0.31
<b>One-way ANOVA</b>		<b>P value</b>	
		0.02*	
<b>Pairwise comparisons</b>		HSD <sub>.05</sub> = 0.5934 HSD <sub>.01</sub> = 0.7607	Q <sub>.05</sub> = 3.5064 Q <sub>.01</sub> = 4.4948
<b>Group A:Group B</b>	M <sub>1</sub> = 1.01 M <sub>2</sub> = 0.37	0.64	Q=3.85 (p=0.02940)*
<b>Group A:Group C</b>	M <sub>1</sub> = 1.01 M <sub>3</sub> = 0.85	0.16	Q=0.96 (p=0.77643)
<b>Group B:Group C</b>	M <sub>2</sub> = 0.37 M <sub>3</sub> = 0.85	0.49	Q=2.88 (p=0.12223)

recovery). The group with the appliance alone (Group B) showed significantly lower gene expression compared to the control group (Group A), but no significant difference is observed between the control group and the group with both the appliance and recovery (Group C). These results suggest that the recovery intervention might have mitigated the decrease in myostatin gene expression observed with the appliance alone.

### B) Lateral pterygoid muscle

The impact of interventions on myostatin gene expression in the lateral pterygoid muscle was investigated and following results were observed (Fig. 4, Table 3).

The mean gene expression levels for myostatin levels in lateral pterygoid muscle were as follows:

- Group A (Control): Mean  $\pm$  SD = 1.34 $\pm$ 1.35
- Group B (Appliance): Mean  $\pm$  SD = 0.47 $\pm$ 0.06
- Group C (Appliance + Recovery): Mean  $\pm$  SD = 0.70 $\pm$ 0.40

The ANOVA test indicated a significant difference between the groups ( $p=0.002^*$ ), suggesting that at least one of the groups had a different mean gene expression level compared to the others. Upon applying pairwise comparisons:

- Group A vs. Group B: A significant difference was found in gene expression levels ( $p=0.00206$ ) between these two groups. Group A had a higher mean gene expression compared to Group B.
- Group A vs. Group C: A significant difference was found in gene expression levels ( $p=0.02462$ ) between these two groups. Group A had a higher mean gene expression compared to Group C.
- Group B vs. Group C: No significant difference was found in gene expression levels ( $p=0.57792$ ) between these two groups.

Myostatin gene expression in the lateral pterygoid muscle appears to be influenced by the intervention (appliance and recovery). Both the appliance alone (Group B) and the appliance with recovery (Group C) groups show lower gene expression compared to the control group (Group A), but there is no significant difference in gene expression between these two groups. These findings suggest that the appliance intervention might be associated with a decrease in myostatin gene expression in the lateral pterygoid muscle, and the addition of recovery does not significantly alter this effect.

**Table 3.** Mean fold change values of myostatin expression in lateral pterygoid muscle. A one-way ANOVA based on ranks test was done to compare between three groups of lateral pterygoid muscle for myostatin gene expression (\*the result was significant at  $p<0.05$ ). Pairwise comparisons with Tukey's HSD for myostatin gene expression in the lateral pterygoid muscle, showcasing mean differences, critical values (HSD), and  $p$ -values for significant group distinctions ( $*p<0.05$ ). SD: standard deviation, n: sample size

S.No.	Group A (Control) n=10	Group B (Appliance) n=10	Group C (Appliance+ Recovery) n=10
1	0.7	0.48	1.27
2	0.64	0.47	0.72
3	3.37	0.4	0.44
4	1.16	0.54	0.38
5	0.94	0.24	0.5
6	0.99	0.45	0.89
7	1.61	0.94	1.2
8	1.55	0.76	0.34
9	1.19	0.32	0.45
10	1.26	0.13	0.84
<b>Mean<math>\pm</math>SD</b>	1.34 $\pm$ 1.35	0.47 $\pm$ 0.06	0.7 $\pm$ 0.40
<b>One-way ANOVA</b>		<b>P value</b> 0.002*	
<b>Pairwise comparisons</b>		HSD <sub>.05</sub> = 0.5654 HSD <sub>.01</sub> = 0.7248	Q <sub>.05</sub> = 3.5064 Q <sub>.01</sub> = 4.4948
<b>Group A:Group B</b>	M <sub>1</sub> = 1.34 M <sub>2</sub> = 0.47	0.87	Q=5.38 ( $p=0.00206$ )*
<b>Group A: Group C</b>	M <sub>1</sub> = 1.34 M <sub>3</sub> = 0.70	0.64	Q=3.96 ( $p=0.02462$ )*
<b>Group B: Group C</b>	M <sub>2</sub> = 0.47 M <sub>3</sub> = 0.70	0.23	Q=1.43 ( $p=0.57792$ )

### Comparison of myostatin expression in masseter and lateral pterygoid muscles

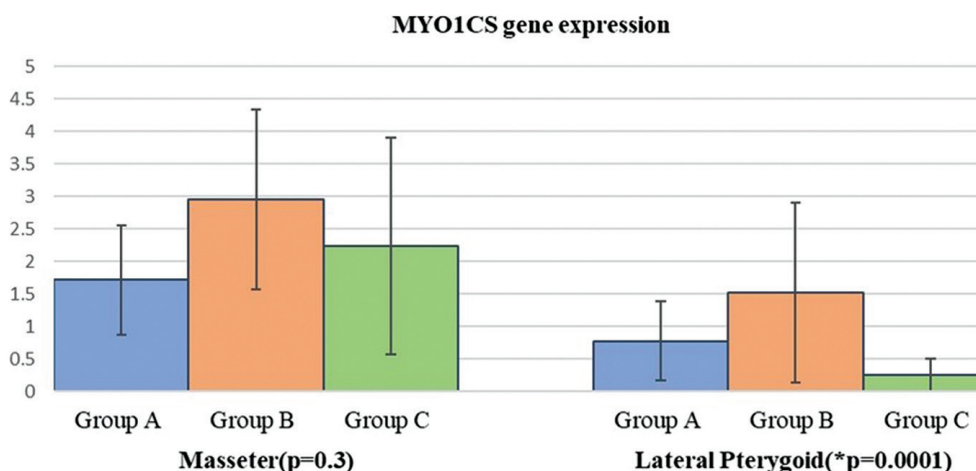
The myostatin gene expression in the masseter muscle was influenced by the intervention, with the group undergoing both appliance and recovery showing no significant difference compared to the control group. However, the group with only the appliance exhibited a significantly lower myostatin gene expression. These findings suggest that the recovery intervention might have mitigated the decrease in myostatin gene expression observed with the appliance alone. In the lateral pterygoid muscle, both the appliance alone and the appliance with recovery groups showed

significantly lower myostatin gene expression compared to the control group. However, there was no significant difference between the appliance alone and the appliance with recovery groups.

### MYO1C gene expression in masticatory muscles

#### Masseter muscle

The impact of interventions on MYO1C expression in the masseter muscle was investigated and the following results were observed (Fig. 5, Table 4).



**Figure 5.** Bar graph illustrating MYO1C gene expression levels in the masseter and lateral pterygoid muscles. The data, derived from a sample size of 10 for each of the three groups, was analyzed using One-way ANOVA, and significant differences are denoted by (\* $p < 0.05$ ).

**Table 4** Mean fold change values of MYO1C expression in masseter muscle. A one-way ANOVA based on ranks test was done to compare between three groups of masseter muscle for MYO1C gene expression (\*the result was significant at  $p < 0.05$ ). SD: standard deviation, n: sample size

S.No.	Group A (Control) n=10	Group B (Appliance) n=10	Group C (Appliance+ Recovery) n=10
1	2.82	8.9	1.08
2	3.74	1.3	6.18
3	0.14	1.16	1.38
4	0.15	0.46	0.33
5	0.45	0.88	1.45
6	2.03	2.56	1.84
7	1.94	1.99	3.68
8	1.76	1.59	2.82
9	1.46	5.99	1.83
10	2.57	4.76	1.79
<b>Mean±SD</b>	1.71±0.85	2.96±1.38	2.24±1.66
<b>One-way ANOVA</b>		<b>P value</b>	
		0.3	

The mean gene expression levels for MYO1C levels in the masseter muscle were as follows:

- Group A (Control): Mean  $\pm$  SD = 1.71 $\pm$ 0.85
- Group B (Appliance): Mean  $\pm$  SD = 2.96 $\pm$ 1.38
- Group C (Appliance + Recovery): Mean  $\pm$  SD = 2.24 $\pm$ 1.66

The ANOVA test revealed no significant difference between the groups ( $p=0.3$ ), implying that the mean MYO1C expression levels in the masseter muscle are not significantly different between the control group and the groups that received the appliance intervention with or without recovery. These findings suggest that the appliance intervention and the addition of a recovery phase may have no effect on MYO1C expression in the masseter muscle. Further research may be needed to explore other factors that could potentially influence MYO1C expression in the masseter muscle or to confirm these findings with a larger sample size or different experimental conditions.

### Lateral pterygoid muscle

The impact of interventions on MYO1C gene expression in the lateral pterygoid muscle was investigated and the following results were observed (Fig. 5, Table 5).

The mean gene expression levels for MYO1C levels in the lateral pterygoid muscle were as follows:

- Group A (Control): Mean  $\pm$  SD = 0.78 $\pm$ 0.60
- Group B (Appliance): Mean  $\pm$  SD = 1.52 $\pm$ 1.38
- Group C (Appliance + Recovery): Mean  $\pm$  SD = 0.26 $\pm$ 1.25

The ANOVA test indicated a significant difference between the groups ( $p=0.0001^*$ ), suggesting that the mean MYO1C expression levels in the lateral pterygoid muscle was significantly different across the control group and the groups undergoing the appliance intervention with or without recovery. Upon applying pairwise comparisons:

- Group A vs. Group B: A significant difference was found in the MYO1C expression levels ( $p=0.01637$ ) between these two groups. Group B had a higher mean MYO1C expression compared to Group A.
- Group A vs. Group C: No significant difference was observed in the MYO1C expression levels ( $p=0.10469$ ) between these two groups.
- Group B vs. Group C: No significant difference was observed in the MYO1C expression levels ( $p=0.00007$ ) between these two groups.

Based on the provided data and statistical analysis,

**Table 5.** Mean fold change values of MYO1C expression in the lateral pterygoid muscle. One-way ANOVA based on ranks test was done to compare between three groups of lateral pterygoid muscle for MYO1C gene expression (\*the result was significant at  $p<0.05$ ). Pairwise comparisons with Tukey's HSD for MYO1C gene expression in the lateral pterygoid muscle, showcasing mean differences, critical values (HSD), and  $p$ -values for significant group distinctions ( $*p<0.05$ ). SD: standard deviation, n: sample size

S. No.	Group A (Control) n=10	Group B (Appliance) n=10	Group C (Appliance+ Recovery) n=10
1	0.42	1.5	0.6
2	0.63	3.46	0.08
3	1.67	0.31	0.07
4	0.4	0.81	0.26
5	1.14	1.95	0.28
6	0.49	1.63	0.34
7	0.92	1.78	0.39
8	0.74	0.92	0.12
9	0.93	1.71	0.1
10	0.49	1.11	0.35
<b>Mean<math>\pm</math>SD</b>	0.78 $\pm$ 0.60	1.52 $\pm$ 1.38	0.26 $\pm$ 1.25
<b>One-way ANOVA</b>		<b>P value</b>	
		0.0001*	
<b>Pairwise Comparisons</b>		HSD <sub>.05</sub> = 0.6130	Q <sub>.05</sub> = 3.5064
		HSD <sub>.01</sub> = 0.7858	Q <sub>.01</sub> = 4.4948
<b>Group A:Group B</b>	M <sub>1</sub> = 0.78 M <sub>2</sub> = 1.52	0.73	Q=4.20 ( $p=0.01637$ )*
<b>Group A:Group C</b>	M <sub>1</sub> = 0.78 M <sub>3</sub> = 0.26	0.52	Q=3.00 ( $p=0.10469$ )
<b>Group B:Group C</b>	M <sub>2</sub> = 1.52	1.26	Q=7.20 ( $p=0.00007$ )*

MYO1C expression in the lateral pterygoid muscle appeared to be influenced by the appliance intervention. The addition of the recovery phase had no significant effect on MYO1C expression levels when compared to either the control or appliance-alone groups.

### Comparison of MYO1C expression in the masseter and lateral pterygoid muscles

MYO1C expression levels in the masseter muscle did not show significant differences across the control group and the groups undergoing the appliance intervention with or without recovery. In contrast, MYO1C expression levels in the lateral pterygoid muscle exhibited significant differences across the groups. The appliance-alone group (Group B) showed significantly higher MYO1C expression compared to both the control group (Group A) and the appliance with recovery group (Group C). However, there was no signifi-

cant difference between Group A and Group C.

### Histological analysis

The tissue samples were analyzed using periodic acid Schiff staining and the following observations were noted.

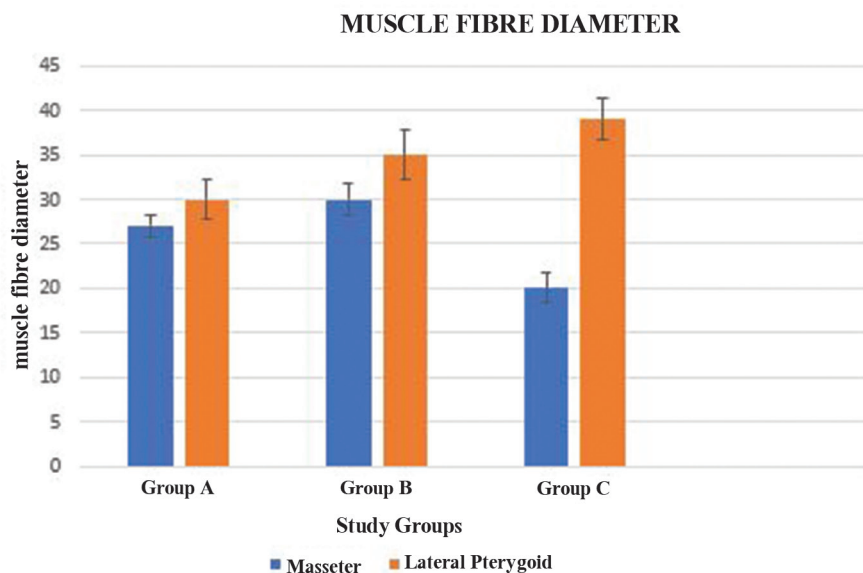
#### Masseter muscle

The muscle fiber diameter was measured and following observations were noted (Figs 6, 7, Table 6).

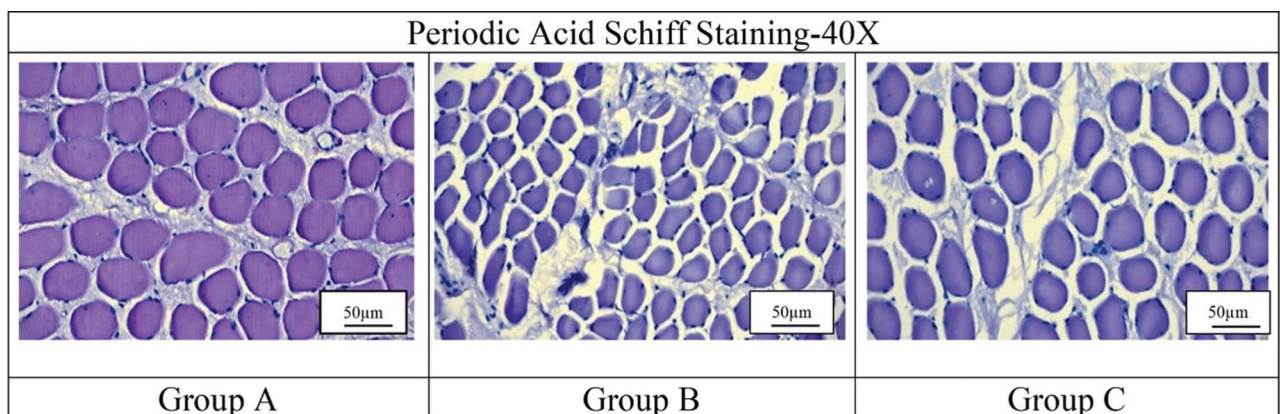
#### Masseter muscle fiber diameter

The data presents the mean  $\pm$  standard deviation (SD) muscle fiber diameter in the masseter muscle across three groups: Control (Group A), Appliance (Group B), and Appliance + Recovery (Group C).

Group A (Control): Mean  $\pm$  SD =  $27 \pm 1.31 \mu\text{m}$



**Figure 6.** Bar graph illustrating mean values of the masseter and lateral pterygoid muscle fiber diameter in Groups A, B, and C. Error bars represent standard error of deviation. An analysis of variance (ANOVA) was conducted, revealing a significant difference in muscle fiber diameter among the groups ( $p < 0.05$ ).



**Figure 7.** Periodic Acid Schiff staining of masseter muscle. 40 $\times$  magnification.

**Table 6.** Mean fiber diameter of masseter muscle in Groups A, B, and C. Values are expressed in micrometers ( $\mu\text{m}$ ) and are represented as mean  $\pm$  standard deviation (SD). An analysis of variance (ANOVA) was conducted, revealing a significant difference in muscle fiber diameter among the groups ( $*p<0.05$ ). Group comparisons were performed using Tukey's post hoc test. n: sample size.

S. No.	Group A (Control) n=10	Group B (Appliance) n=10	Group C (Appliance+ Recovery) n=10
1	25	32	18
2	27	31	20
3	26	32	19
4	28	30	23
5	25	28	17
6	29	29	19
7	28	31	21
8	29	27	22
9	27	28	20
10	26	32	21
Mean Muscle Fiber diameter $\pm$ SD	27 $\pm$ 1.31	30 $\pm$ 1.75	20 $\pm$ 1.68
<b>One-way ANOVA</b>		<b>P value</b>	
		0.00012*	
<b>Pairwise Comparisons</b>		HSD <sub>.05</sub> = 1.9324 HSD <sub>.01</sub> = 2.4771	Q <sub>.05</sub> = 3.5064 Q <sub>.01</sub> = 4.4948
<b>Group A:Group B</b>	Group A = 27.00 Group B = 30.00	3	Q=5.44 ( $p=0.00185$ )*
<b>Group A:Group C</b>	Group A = 27.00 Group C = 20.00	7	Q=12.70 ( $p=0.00000$ )*
<b>Group B:Group C</b>	Group B = 30.00 Group C = 20.00	10	Q=18.15 ( $p=0.00000$ )*

Group B (Appliance): Mean  $\pm$  SD =30 $\pm$ 1.75  $\mu\text{m}$

Group C (Appliance + Recovery): Mean $\pm$ SD =20 $\pm$ 1.68  $\mu\text{m}$

The ANOVA test indicated a significant difference in mean muscle fiber diameter among the three groups, with a  $p$ -value of 0.00012. Upon applying pairwise comparisons:

- Group A vs. Group B: There is a significant difference in mean muscle fiber diameter between these two groups ( $p=0.00185$ ), with Group B having a higher mean fiber diameter compared to Group A.
- Group A vs. Group C: There is a significant difference in the mean muscle fiber diameter between these two groups ( $p=0.00000$ ), with Group C having a lower mean fiber diameter compared to Group A.
- Group B vs. Group C: There is a significant difference in the mean muscle fiber diameter between these two groups ( $p=0.00000$ ), with Group B having a higher mean fiber diameter compared to Group C.

The data suggests that the intervention, whether it involves appliance usage alone (Group B) or appliance usage with a recovery protocol (Group C), has a significant effect on the mean muscle fiber diameter in the masseter muscle

compared to the control group (Group A). Group B, which underwent the appliance intervention, exhibited a higher mean muscle fiber diameter compared to both the control and appliance + recovery groups. Conversely, Group C, which received the appliance + recovery intervention, showed a lower mean muscle fiber diameter compared to both the control and appliance-alone groups.

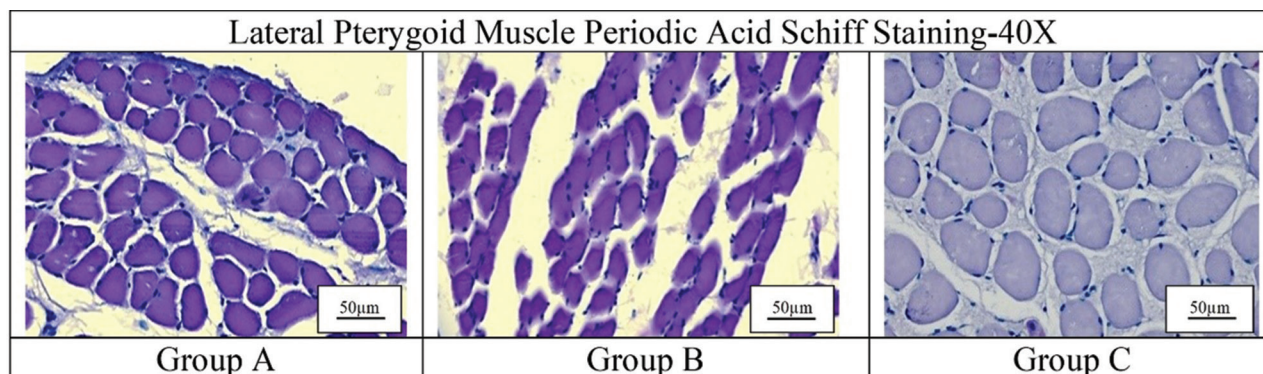
### **Lateral pterygoid muscle**

The muscle fiber diameter was measured and the following observations were noted (Figs 6, 8; Table 7).

### **Lateral pterygoid muscle fiber diameter**

The data presents the mean  $\pm$  standard deviation (SD) muscle fiber diameter in the lateral pterygoid muscle across three groups: Control (Group A), Appliance (Group B), and Appliance + Recovery (Group C).

- Group A (Control): Mean $\pm$ SD =30 $\pm$ 2.16  $\mu\text{m}$
- Group B (Appliance): Mean $\pm$ SD =35 $\pm$ 2.78  $\mu\text{m}$
- Group C (Appliance + Recovery): Mean  $\pm$  SD=39 $\pm$ 2.3  $\mu\text{m}$



**Figure 8.** Periodic Acid Schiff staining of lateral pterygoid muscle. 40× magnification.

**Table 7.** Mean fiber diameter of lateral pterygoid muscle in Groups A, B, and C. Values are expressed in micrometers (µm) and are represented as mean ± standard deviation (SD). An analysis of variance (ANOVA) was conducted, revealing a significant difference in muscle fiber diameter among the groups (\**p*<0.05). Group comparisons were performed using Tukey’s post hoc test. n: sample size

S. No.	Group A (Control) n=10	Group B (Appliance) n=10	Group C (Appliance+ Recovery) n=10
1	31	30	39
2	32	33	40
3	27	34	41
4	30	35	42
5	28	36	38
6	33	38	35
7	31	32	39
8	29	37	37
9	32	35	42
10	27	40	37
Mean Muscle Fiber Diameter±SD	30±2.16	35±2.78	39±2.3
<b>One-way ANOVA</b>		<b>P value</b>	
		0.0009*	
<b>Pairwise Comparisons</b>		HSD <sub>.05</sub> = 2.6992	Q <sub>.05</sub> = 3.5064
		HSD <sub>.01</sub> = 3.4601	Q <sub>.01</sub> = 4.4948
<b>Group A:Group B</b>	Group A = 30.00 Group B = 35.00	5	Q=6.50 (p=0.00026)*
<b>Group A:Group C</b>	Group A = 30.00 Group C = 39.00	9	Q=11.69 (p=0.00000)*
<b>Group B:Group C</b>	Group B = 35.00 Group C = 39.00	4	Q=5.20 (p=0.00290)*

The ANOVA test indicated a significant difference in mean muscle fiber diameter among the three groups, with a *p*-value of 0.0009. Upon applying pairwise comparisons:

- Group A vs. Group B: There is a significant difference in mean muscle fiber diameter between these two groups (*p*=0.00026), with Group B having a higher mean fiber diameter compared to Group A.
- Group A vs. Group C: There is a significant difference in mean muscle fiber diameter between these two groups (*p*=0.00000), with Group C having a higher mean fiber diameter compared to Group A.
- Group B vs. Group C: There is a significant difference in mean muscle fiber diameter between these two groups (*p*=0.00290), with Group C having a higher mean fiber diameter compared to Group B.

The data suggests that the intervention, whether it involves appliance usage alone (Group B) or appliance usage with a recovery protocol (Group C), has a significant effect on the mean muscle fiber diameter in the lateral pterygoid muscle compared to the control group (Group A). Both Group B and Group C exhibited higher mean muscle fiber diameters compared to the control group, with Group C showing the highest mean diameter.

### Comparison between masseter and lateral pterygoid muscles

Both muscles responded positively to the intervention (appliance), showing an increase in mean muscle fiber diameter compared to the control group. However, the response to the combined intervention (appliance + recovery) differed between the two muscles. While the masseter muscle showed a decrease in mean muscle fiber diameter, the lateral pterygoid muscle showed a further increase. This suggests that the addition of recovery to the intervention may have different effects on different muscles, possibly due to variations in muscle function, structure, or response to the intervention (Fig. 5).

### Discussion

The changes in gene expression of the two genes myostatin and MYO1C were assessed in the masseter and lateral pterygoid muscles after placing a mandibular advancement appliance. Wistar rats were chosen as an experimental animal model, as they possess genetic homogeneity, mitigating individual variations often encountered in clinical studies. Despite anatomical differences, rats share key similarities with humans in muscle biology. Both species exhibit conserved pathways regulating skeletal muscle adaptation (e.g., myostatin, IGF-1, satellite cells), comparable roles of the masseter and lateral pterygoid in mandibular function, and structural changes in response to mandibular advancement. Their masticatory muscles also display reversible plasticity during recovery phases and contain both type I and type II fibers that adapt differently to mechanical loading. These parallels support the translational value of our findings to human orthodontic contexts. They offer advantages in terms of ease of handling and cost-effectiveness compared to larger animals.<sup>[17]</sup> The selection of 6–8-week-old male Wistar rats as experimental animals was critical, as they enter puberty around the 6–8-week mark and have less hormonal variation compared to females.<sup>[18]</sup> This hormonal stability reduces potential confounding factors in studies, where hormonal fluctuations might impact experimental outcomes even when the same stimulus is applied.

A recover removal of the appliance in the experimental group allows us to assess the stability and durability of changes induced by the appliance. It provides a window to observe whether the effects observed during the appliance application persist, regress, or stabilize after its removal.

The study aimed to simulate an intervention relevant to the mandibular area; placing the appliance there would directly target this anatomical region.<sup>[13]</sup> Practical considerations, such as ease of appliance placement and monitoring, favored the mandibular jaw. This was confirmed from our pilot study wherein the appliance was placed on both the upper and lower jaws, but it was found that the retention of the appliance was better on the lower jaw. Accessibility and feasibility of applying and maintaining the appliance on the lower jaw influenced this decision.

Body weight was monitored throughout the study to assess the effects of the mandibular advancement appliance. Initially, a minor, statistically insignificant weight loss was observed in both control and experimental groups, attributed to adjustment to the appliance. Importantly, this effect was transient, as weights in the experimental group steadily increased thereafter, ultimately approaching control values by the study's end. This trend suggests that the appliance did not cause long-term nutritional compromise or systemic stress and that the animals adapted well over time. Thus, body weight changes appear to reflect a temporary acclimatization rather than an adverse effect of the intervention.

An anterior crossbite was noted upon appliance removal, which persisted during the recovery phase. These findings suggested that rats can adapt to the appliance without significant long-term weight changes, with any initial effects being reversible upon appliance removal.

TaqMan assays were chosen as they are known for their high specificity, as they use dual-labeled probes targeting a specific sequence within the PCR product. This reduces the chances of nonspecific amplification. In our experiment with multiple potential targets, TaqMan assays offered better specificity and reliability in distinguishing between different targets.<sup>[19,20]</sup>

Our primary endpoints were transcriptional changes measured by quantitative PCR and RNA-seq, offering highly sensitive, quantitative data that directly reflect gene-expression levels. While IHC provides spatial context, its semi-quantitative nature and dependence on antibody validation introduce variability. Transcriptomic data present clearer fold-change metrics. Validating multiple antibodies for our panel of target proteins was beyond our logistical scope for this study. Prioritizing molecular assays enabled timely completion within our funding and timeline.

Following the histological analysis of the muscles, the masseter muscle and the lateral pterygoid muscle exhibit distinct responses to the intervention (appliance) and the combined intervention with recovery. In the masseter muscle, appliance usage (Group B) led to a significant increase in mean muscle fiber diameter compared to the control group (Group A), suggesting a potential hypertrophic effect. However, when the recovery protocol was added (Group C), the mean muscle fiber diameter decreased significantly compared to both the control and appliance-alone groups. This suggests that the recovery

intervention might have mitigated the hypertrophic effect observed with the appliance alone. In contrast, the lateral pterygoid muscle responded differently to the interventions. Both appliance usage alone (Group B) and appliance usage combined with recovery (Group C) led to a significant increase in mean muscle fiber diameter compared to the control group (Group A). Moreover, the lateral pterygoid muscle in Group C exhibited the highest mean muscle fiber diameter among all groups. These results suggest that both interventions had a hypertrophic effect on the lateral pterygoid muscle, with the recovery protocol possibly enhancing this effect. These findings highlight the importance of considering muscle-specific responses when designing interventions or treatments aimed at modifying muscle characteristics or function.

Histopathological analysis has revealed an increase in overall cartilage thickness upon placement of the mandibular advancement bite-jumping appliance, consistent with previous studies<sup>[10-12,14]</sup>, suggesting enhanced cartilage formation due to the appliance. We conducted molecular analysis of condylar cartilage using real-time RT-PCR and histological assessments. Our results revealed significant genetic expression changes in both experimental groups, specifically for *Sdf1* ( $p < 0.05$ ) and *Foxc1* ( $p < 0.05$ ). Histological investigations showed a marked increase in the proliferative and hypertrophic layers of condylar cartilage. These findings suggest that mandibular advancement bite-jumping appliances induce proliferative and hypertrophic changes in the cartilage, characterized by elevated *Foxc1* levels and decreased *Sdf1* levels. Notably, persistent gene expression post-appliance removal indicates sustained joint stimulation.<sup>[21]</sup>

Myostatin, a member of the transforming growth factor-beta (TGF- $\beta$ ) superfamily, has garnered attention as a critical negative regulator of muscle growth.<sup>[22-25]</sup> Its role in modulating muscle mass and regulating myogenesis has been extensively documented<sup>[26,27]</sup>, suggesting its potential involvement in responding to orthodontic stimuli and influencing muscle adaptations during treatment<sup>[28,29]</sup>. The masseter muscle and the lateral pterygoid muscle exhibited different responses to the intervention. While the appliance alone led to a decrease in myostatin gene expression in both muscles, the addition of recovery had varying effects. In the masseter muscle, the recovery intervention seemed to counteract the decrease in myostatin gene expression observed with the appliance alone, indicating a potential protective effect. In contrast, in the lateral pterygoid muscle, the recovery intervention did not significantly alter the decrease in myostatin gene expression induced by the appliance alone. These findings suggest that interventions targeting myostatin gene expression may have muscle-specific effects. Understanding these differential responses is crucial for designing targeted interventions for specific muscles. The differential responses observed in the masseter and lateral pterygoid muscles may have implications for treatments targeting muscle function, such as in conditions like temporomandibular disorders or orthodontic

treatments. It has been confirmed from previous studies on different groups of muscles that downregulation of myostatin results in muscle hypertrophy, while upregulation will result in muscle atrophy.<sup>[30-32]</sup> The results of our study are consistent with these findings. Though there are no previous studies evaluating the changes in myostatin after placement of a functional appliance, various studies assessing the expression of gene levels in subjects with malocclusion<sup>[33]</sup> or with a different muscle group have been done for myostatin. Further research is needed to elucidate the underlying mechanisms driving the differential responses in myostatin gene expression between the masseter and lateral pterygoid muscles.

MYO1C, an isoform of the myosin superfamily, stands out for its significant role in modulating muscle contractility and cytoskeletal dynamics.<sup>[34-37]</sup> Studies have highlighted its involvement in regulating actin networks and its potential responsiveness to mechanical stimuli<sup>[38-40]</sup>, positioning it as a key candidate in exploring the effects of orthodontic appliances on masticatory muscle<sup>[41]</sup>. The comparison of MYO1C expression in the masseter and lateral pterygoid muscles reveals distinct patterns of response to the intervention. In the masseter muscle, MYO1C expression levels were not significantly altered by the appliance intervention or the addition of recovery. This suggests that MYO1C may not play a significant role in the response of the masseter muscle to the intervention studied. In contrast, MYO1C expression in the lateral pterygoid muscle was significantly influenced by the appliance intervention. Specifically, the appliance-alone group exhibited higher MYO1C expression compared to both the control group and the appliance with recovery group. These findings suggest muscle-specific responses of MYO1C expression to the intervention studied. Understanding these differences is crucial for targeting interventions effectively and developing personalized treatment approaches for conditions affecting this region.

The differential response between the two muscles may be attributed to their distinct roles and loading patterns. The masseter muscle showed reduced fiber diameter during recovery as its mechanical demand normalized. In contrast, the lateral pterygoid, which plays a key role in mandibular protrusion and stabilization, continued to hypertrophy, likely reflecting sustained loading and a higher proportion of load-responsive fibers. These morphological changes are consistent with gene expression patterns: myostatin, a negative regulator of growth, and MYO1C, a mechanosensitive cytoskeletal regulator, likely act in coordination to mediate remodeling under altered loading. The differential regulation of these pathways may contribute to the distinct responses observed between the two muscles. While additional signaling mechanisms such as IGF-1 and myogenic regulatory factors are also known to influence muscle adaptation, our focus on myostatin and MYO1C highlights their key mechanosensitive roles, with future work needed to place them within a broader regulatory framework.

This pattern not only shows the plasticity of muscle tissue but also emphasizes its inherent regulatory mechanisms, indicative of a dynamic equilibrium that exists to maintain homeostasis.<sup>[42,43]</sup> Though there are no previous studies evaluating the changes in the MYO1C gene expression levels in subjects after placing an orthodontic appliance, its role in maintaining adaptive response in the inner ear is suggestive of its role in maintaining equilibrium between muscles. Further research is warranted to elucidate the functional significance of altered MYO1C expression in the lateral pterygoid muscle and its implications for muscle function and clinical outcomes.

## Clinical relevance: beyond orthodontics – broader impact of our mandibular advancement study

This animal study on mandibular advancement, while rooted in orthodontics, holds valuable implications across several disciplines. Modern dentistry is rapidly incorporating ‘omics’ technologies to tailor interventions. Gene-expression profiles from our study can serve as biomarkers to predict individual tissue responsiveness, enabling customized appliance parameters and improved treatment efficiency. In craniofacial development research, the skeletal and muscular adaptations observed in response to mandibular advancement provide a model for understanding growth modulation in conditions such as micrognathia and syndromic malformations. In sleep medicine, mandibular repositioning is a widely used approach for managing obstructive sleep apnea (OSA), and our findings may inform how orthopedic forces influence oropharyngeal structures and airway dynamics. Temporomandibular joint (TMJ) research also benefits from these insights, as the study captures condylar cartilage responses to postural changes, aiding in understanding joint remodeling processes. From a bone biology perspective, the adaptive responses documented in masticatory muscles and cartilage contribute to broader discussions on mechanotransduction and skeletal remodeling under functional loads. Additionally, pediatric rehabilitation strategies—particularly in patients with neuromuscular or growth disorders—can potentially incorporate mandibular advancement protocols for stimulating targeted growth. Finally, prosthodontists and maxillofacial surgeons may draw from these findings to anticipate muscular and joint adaptations following surgical advancement or mandibular reconstruction, ultimately supporting more stable and biologically informed treatment outcomes.

## Limitations and future directions

While this study provides valuable insights into muscle- and gene-specific responses to mandibular advancement

appliances, several limitations should be acknowledged:

1. Although Wistar rats offer genetic homogeneity and ease of handling, extrapolating findings directly to humans is limited by species-specific differences in muscle biology, growth patterns, and craniofacial biomechanics.
2. The study primarily assessed short- and mid-term effects (during appliance use and one month post-removal). Longer follow-up periods would be necessary to understand the permanence or reversibility of muscular and cartilaginous adaptations over the long term.
3. The investigation focused on two genes, myostatin and MYO1C. However, muscle adaptation involves complex regulatory networks, including other myogenic, mechanotransductive, and inflammatory genes, which were not explored.
4. Despite using genetically similar rats, biological variability can still exist. Larger sample sizes or multiple strains could help confirm the reproducibility and robustness of the findings.
5. In our study we made a deliberate decision to include male subjects exclusively. This decision was based on the desire to minimize biological variability that could arise from hormonal fluctuations, which are particularly pronounced in females due to menstrual cycles and other hormonal factors that can influence gene expression related to bone remodeling, tissue regeneration, and response to mechanical stress, potentially confounding the results and making it challenging to draw clear conclusions regarding the effects of the appliance itself. However, we recognize that females, as future appliance users, must also be considered, and we acknowledge the importance of including female subjects in future studies. This would help to identify any gender-specific differences in gene expression and ensure that our findings are applicable across both sexes.

## Conclusion

In conclusion, our study delves into the intricate molecular dynamics underlying the effects of functional mandibular advancement and subsequent recovery on gene expression in masticatory muscles of young male Wistar rats. The observed differential responses in gene expression between the masseter and lateral pterygoid muscles underscore the complexity of muscle physiology and highlight the importance of muscle-specific considerations in orthodontic interventions. Our findings suggest that interventions targeting myostatin and MYO1C expression may elicit muscle-specific effects, necessitating tailored approaches for optimizing clinical outcomes. Furthermore, our study highlights the adaptive capacity of masticatory muscles in response to orthodontic interventions, as evidenced by the transient nature of observed changes upon appliance removal. This dynamic expression signifies an adaptive mechanism aimed at accommodating altered conditions,

reflecting the responsive nature of muscles to external influences. Our study's unique emphasis on the recovery phase provides a novel perspective, offering insights into the lasting effects of these interventions. In summary, these findings set the stage for future research, guiding the refinement of orthodontic treatments for enhanced efficacy. Our exclusive focus on the recovery phase adds a new dimension to understanding the relationship between appliances and masticatory muscle biology, opening avenues for further exploration in orthodontic research. Further research is warranted to elucidate the underlying mechanisms driving muscle-specific responses and to translate these findings into clinical practice effectively.

## Ethical approval

Ethical approval for this study was granted by the Institutional Animal Ethics Committee (Protocol IAEC/CPC-SEA/013/2022).

## Ethical statements

- The authors declared that no clinical trials were used in the present study.
- The authors declared that no experiments on humans or human tissues were performed for the present study.
- The authors declared that no informed consent was obtained from the humans, donors or donors' representatives participating in the study. – Not applicable
- The authors declared that they performed certain experiments on animals for the present study for which they were given permission by the Institutional Animal Ethics Committee, and that all procedures adhered to ethical animal research guidelines, including the ARRIVE guidelines, and were approved by the local Ethics Committee.
- The authors declared that no commercially available immortalized human and animal cell lines were used in the present study.

## Conflict of interest

The authors of this manuscript affirm that they have no financial or personal affiliations with individuals or organizations that might unduly affect the results of this work.

## Use of AI

No use of AI was reported.

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## Author contributions

SS: methodology, data curation, writing—original draft; AP: formal analysis, validation, conceptualization, project administration; SB: resources, supervision, funding acquisition, writing—review and editing; ShS: investigation, software, visualization; MG: data curation, validation, writing—review and editing.

## Data availability

All data used are referenced or included in the article.

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