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Effects of age and diet consistency on the expression of myosin heavy-chain isoforms on jaw-closing and jaw-opening muscles in a rat model

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Abstract

Background: Skeletal craniofacial morphology can be influenced by changes in masticatory muscle function, which may also change the functional profile of the muscles.

Objectives: To investigate the effects of age and functional demands on the expression of Myosin Heavy-Chain (MyHC) isoforms in representative jaw-closing and jaw-opening muscles, namely the masseter and digastric muscles respectively.

Methods: Eighty-four male Wistar rats were divided into four age groups, namely an immature ($n=12$; 4-week-old), early adult ($n=24$; 16-week-old), adult ($n=24$; 26-week-old) and mature adult ($n=24$; 38-week-old) group. The three adult groups were divided into two subgroups each based on diet consistency; a control group fed a standard (hard) diet, and an experimental group fed a soft diet. Rats were sacrificed, and masseter and digastric muscles dissected. Real-time quantitative polymerase chain reaction was used to compare the mRNA transcripts of the MyHC isoforms—*Myh7* (MyHC-I), *Myh2* (MyHC-IIa), *Myh4* (MyHC-IIb) and *Myh1* (MyHC-IIx)—of deep masseter and digastric muscles.

Results: In the masseter muscle, hypofunction increases Myh1 (26, 38 weeks; $p<.0001$) but decreases Myh4 (26 weeks; $p=.046$) and Myh2 (26 weeks; $p<.0001$) expression in adult rats. In the digastric muscle, hypofunction increases Myh1 expression in the mature adult rats (38 weeks; $p<.0001$), while Myh2 expression decreases in adult rats (26 weeks; $p=.021$) as does Myh4 (26 weeks; $p=.001$). Myh7 expression is increased in the digastric muscle of mature adult rats subjected to hypofunction (38 weeks; $p=<.0001$), while it is very weakly expressed in the masseter.

Conclusion: In jaw-opening and jaw-closing muscles, differences in myosin expression between hard- and soft-diet-fed rats become evident in adulthood, suggesting that long-term alteration of jaw function is associated with changes in the expression of MyHC isoforms and potential fibre remodelling. This may give insight into the role of function on masticatory muscles and the resultant craniofacial morphology.

KEYWORDS
aging, jaw muscles, messenger RNA, myosin heavy chains, rats, soft diet

1 | INTRODUCTION

Skeletal craniofacial morphology and growth can be influenced by changes in masticatory muscle function[1,2] which may also depend on growth and maturation of the craniofacial complex and functional demands changing the functional profile of the muscles.[3–6]

Skeletal muscles are composed of myofibers divided into four different types, classified by their Myosin Heavy Chain (MyHC) expression profile.[7–10] The analysis of this expression profile is often carried out by real-time quantitative polymerase chain reaction (RT-qPCR), a reliable approach to classify the muscle fibre types[11–13] and a viable approach to provide insight into the functional characteristics of muscles.[14]

Different genes code for different MyHC isoforms. Slow-contracting and fatigue-resistant fibres, presenting an oxidative activity, express *Myh7* (MyHC-I). Fast myofibers can be divided into three subtypes: *Myh2* (MyHC-IIa) fibres still show some fatigue resistance; *Myh1* (MyHC-IIx) is an intermediate type; and *Myh4* (MyHC-IIb) are the fastest and most fatigable fibres. *Myh4* fibres present a high glycolytic activity,[3] *Myh2* fibres a combined glycolytic-oxidative activity and *Myh1* fibres a glycolytic activity. Despite this a priori compartmentalized classification, muscle fibres are highly dynamic, can adapt to new functional demands and substrate availability to change their phenotypes by switching different MyHC isoform genes on and off. When subjected to changes in functional demands, the fibre-type composition, distribution and the cross-sectional area can vary[3,4] to optimize muscle function. These parameters may also change during growth and maturation, during which specific demands exerted according to specific functions may guide muscle development.

Several muscles are involved in jaw function, and their temporal development can vary depending on their primary action. In rodents, jaw-opening muscles develop before jaw-closing muscles because of their involvement in suckling and lapping activities.[15] The digastric, a jaw-opening muscle, also helps to stabilize jaw and hyoid bones,[16] in addition to different roles involving the tongue (swallowing, lapping, suckling). Before the weaning phase, it is involved in suckling activities characterized by low amplitude and fast movements. The masseter, a mandibular elevator muscle, develops later and plays a role in the generation of occlusal forces, especially via its deep portion whose fibres have a vertical orientation. Following the weaning phase and coinciding with the beginning of the eruption of the posterior teeth and the occlusal development, it will be submitted to progressive functional demands for mastication.

A change in the consistency of the diet (hard vs. soft diet) can be used to modify the functional demands of the masticatory muscles and in the case of a soft diet, mimic hypofunctional occlusal demands.[5]

As jaw-opening and jaw-closing muscles are antagonistic and differ with regard to their functional profiles during growth and maturation, we hypothesize that:

(i) hypofunction induces changes in the MyHC isoform expression profile in both masseter and digastric muscles;
(ii) MyHC isoform expression differs between both masseter and digastric muscles when subjected to hypofunction, thus reflecting their antagonistic function. We speculate that a soft diet would reduce masticatory demands and powerful muscle contractions required to chew food, thus leading to a decreased activity of the masseter, but increased activity of the digastric muscle as the need for lapping soft food increases.

Thus, the aim of the study was to investigate the effects of age (from immaturity to mature adulthood) and differing functional demands on the expression profile of MyHC isoforms in the masseter and digastric muscles.

2 | MATERIALS AND METHODS

2.1 | Ethics approval

The present experiments were approved by the ethics committee for animal research (numbers: GE/15/20A and GE31) of the Canton of Geneva, Switzerland.

The reporting of the current study adheres to the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments).[17]

2.2 | Sample

Eighty-four male Wistar rats were used for this study, divided into four different groups based on age: an immature group (12 rats, 4-week-old), an early adult group (24 rats, 16-week-old), an adult group (24 rats, 26-week-old) and a mature adult group (24 rats, 38-week-old) (Figure 1). The three adult groups were divided into two subgroups each, based on diet consistency, namely a control group fed a standard (hard) diet, and an experimental group fed during their whole life a soft diet (simulating hypofunction).

All the animals were housed two per cage. All rats spent the first 3 weeks of their life fed by their mother (suckling and weaning phases), and after this point, they were fed either an ordinary rat hard diet in the form of pellets, or a soft diet made up of standardized proportions of the same pellets in powdered form mixed with water, to a ratio of 100 g powder to 100 mL water. Water was ad libitum. The light/dark regime was 12/12 h. After their arrival, all the

animals were kept in quarantine for a period of 1 week. At the age of 4, 16, 26 and 38 weeks, body weight was measured and the animals were subsequently sacrificed. To induce anaesthesia, inhalation of isoflurane (5%) was used before the animals received an intraperitoneal injection of pentobarbital (150 mg/kg, diluted to 200 mg/mL).

After the rats were sacrificed, both deep masseter and anterior digastric muscles were dissected (Figure 2). RT-qPCR analysis was used to compare the mRNA transcripts of the MyHC isoforms—*Myh7* (MyHC-I), *Myh2* (MyHC-IIa), *Myh4* (MyHC-IIb) and *Myh1* (MyHC-IIx)—of deep masseter and digastric muscles in the experimental and control groups.

2.3 | Methods

All the muscle samples were frozen by liquid-nitrogen-cooled isopentane at −140 to −149°C and stored at −80°C. The frozen samples were cut in a cryostat in sections of 200 µm for RNA extraction. Total RNA was extracted using NucleoZOL (Macherey-nagel, Duren, Germany). cDNA was synthesized from 1 µg RNA using qScript cDNA SuperMix (QIAGEN, Beverly, MA). PCR was performed using the StepOne and StepOnePlus Real-Time PCR Systems (Applied Biosystems, California, USA) and PowerUp SYBER Green Master Mix (Life Technologies, Carlsbad, USA). To determine the changes in steady-state, mRNA was quantified relative to a standard curve generated with serial dilutions of a reference cDNA preparation and normalized using Ribosomal Protein Large, PO (RPLPO) mRNA. All experiments were repeated at least twice by the same operator at two different times. RT-qPCR was used to quantify the mRNA transcripts of the MyHC isoforms regardless of the type of fibre in which they were expressed. The abundance of *Myh7* (MyHC-I), *Myh2* (MyHC-IIa), *Myh4* (MyHC-IIb) and *Myh1* (MyHC-IIx) mRNA relative to the house-keeping gene Ribosomal Protein Large, PO (RPLPO) mRNA in the masseter and digastric muscles of young (4 weeks), early adults (16 weeks), adult (26 weeks) and mature adult (38 weeks) rats was calculated. Primers used for RT-qPCR are the same as those previously described in another study.[18]

2.4 | Statistical analysis

Statistical analysis was performed using GraphPad PRISM (version 9.3.0463). Two-way ANOVA tests, including both age and diet consistency, were used to detect differences between the groups followed by post hoc Tukey's multiple comparison test. *p*-values of $<.05$ were considered statistically significant.

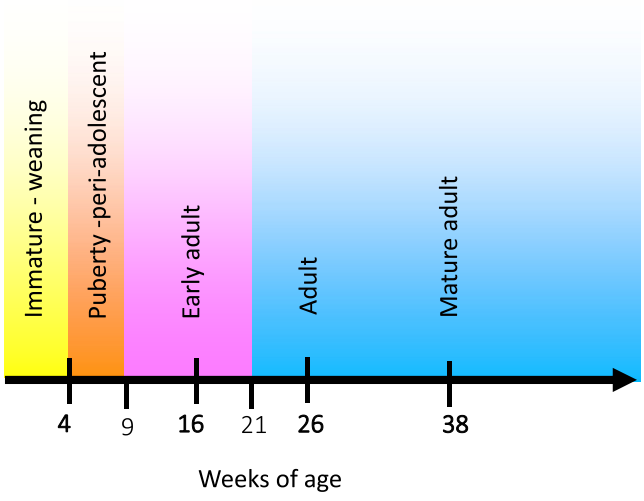

FIGURE 1 Different age groups used in the present study showing the corresponding maturation stages in the rat model.[50,51]

3 | RESULTS

No significant differences in body weight were found between the rats fed a soft diet and those fed a hard diet, for any of the age groups investigated. The results concerning MyHC isoforms in relation to age and functional demands are detailed below.

3.1 | Myosin heavy-chain isoform expression related to age in normally fed rats

Rats fed a standard hard diet showed no significant changes in *Myh1* and *Myh7* expression with age (Figures 3 and 6), and minimal changes in *Myh2* expression with age (Figure 4) in both masseter and digastric muscles. *Myh4* expression increased significantly from the

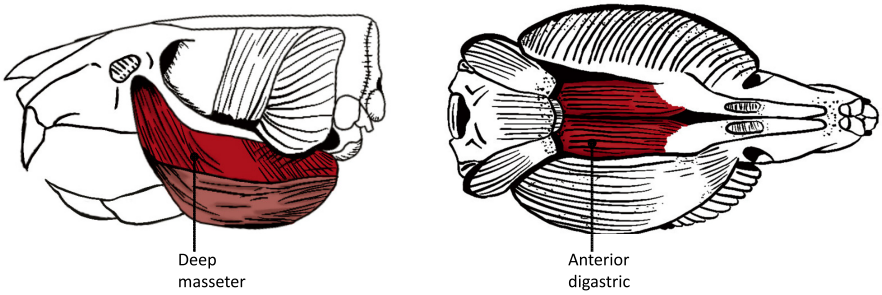

FIGURE 2 Anatomical illustration of the muscles dissected in the current study (deep masseter and anterior digastric muscles).

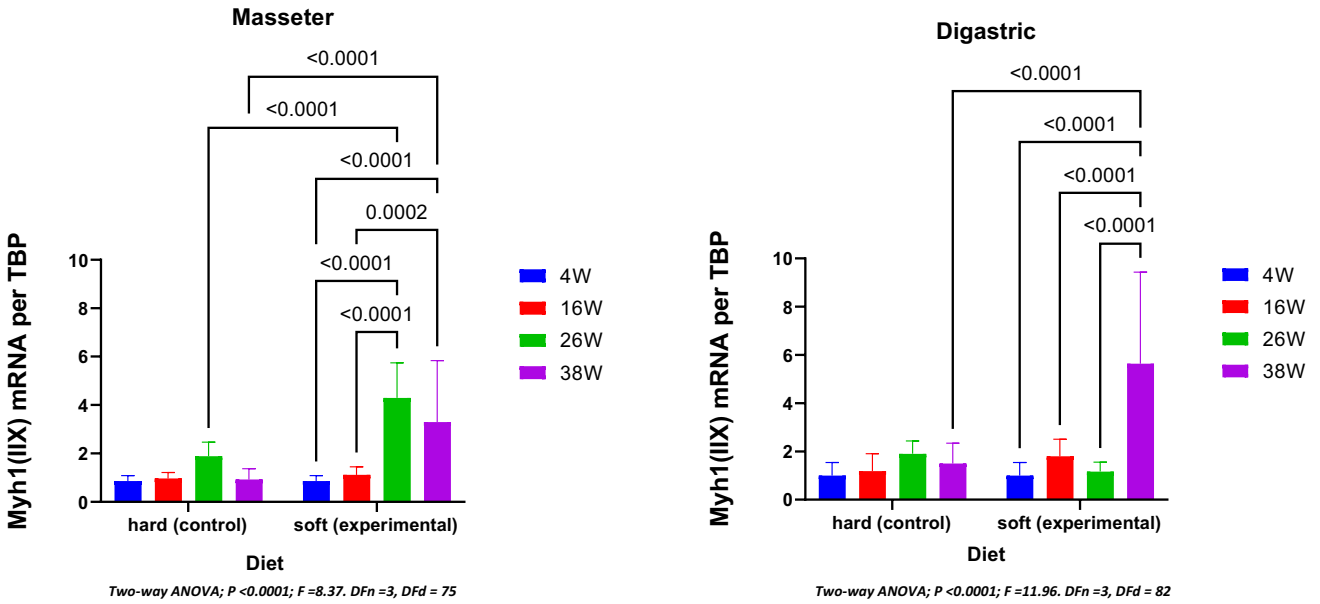

FIGURE 3 mRNA expression for *Myh1* at different time points for both experimental conditions for the masseter and digastric muscles. Only significant *p* values for post-hoc comparisons are shown. TATA binding protein; W, weeks of age.

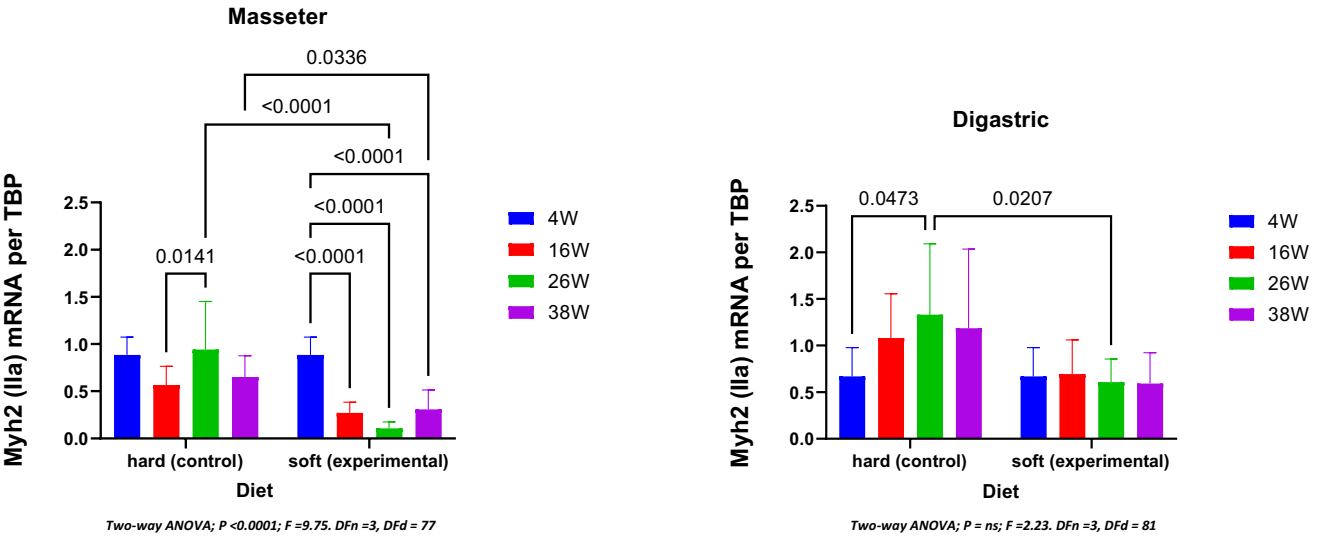

FIGURE 4 mRNA expression for *Myh2* at different time points for both experimental conditions for the masseter and digastric muscles. Only significant p values for post-hoc comparisons are shown. TBP, TATA binding protein; W, weeks of age.

age of 4 to 16 weeks in the masseter muscle ($p < .0001$) (Figure 5), with a gradual decrease thereafter. In the digastric muscle, *Myh4* expression increased at 26 weeks ($p < .0001$) (Figure 5).

3.2 | Myosin heavy-chain isoform expression related to age in rats with masticatory hypofunction

In the masseter muscle of the experimental rats, hypofunction decreased *Myh2* expression from 16 weeks of age onwards ($p < .0001$) (Figure 4) while increasing *Myh1* expression from 26 weeks of age onwards ($p < .0001$) (Figure 3). *Myh4* expression increased transiently in the masseter muscles at 16 weeks of age ($p = .0010$), before decreasing at later time points (Figure 5). *Myh7* was very weakly expressed at all ages in the masseter muscle (Figure 6).

On the other hand, in the digastric muscle of rats with experimental hypofunction, *Myh1* and *Myh7* expression increased only at 38 weeks of age ($p < .0001$; $p < .0001$) (Figures 3 and 6). During maturation, *Myh4* expression increased transiently in the digastric muscles at 16 weeks of age ($p < .0001$), before decreasing at later time points (Figure 5). No significant differences were seen in *Myh2* expression with age in the digastric muscle (Figure 4).

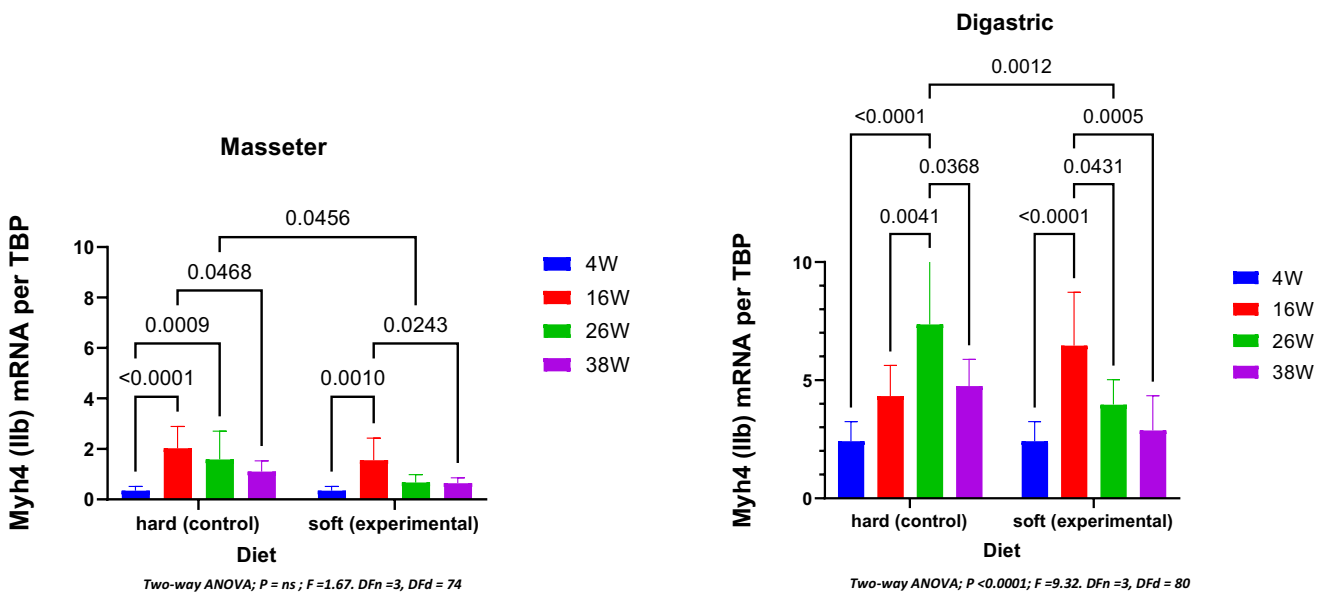

FIGURE 5 mRNA expression for *Myh4* at different time points for both experimental conditions for the masseter and digastric muscles. Only significant p values for post-hoc comparisons are shown. TBP, TATA binding protein; W, weeks of age.

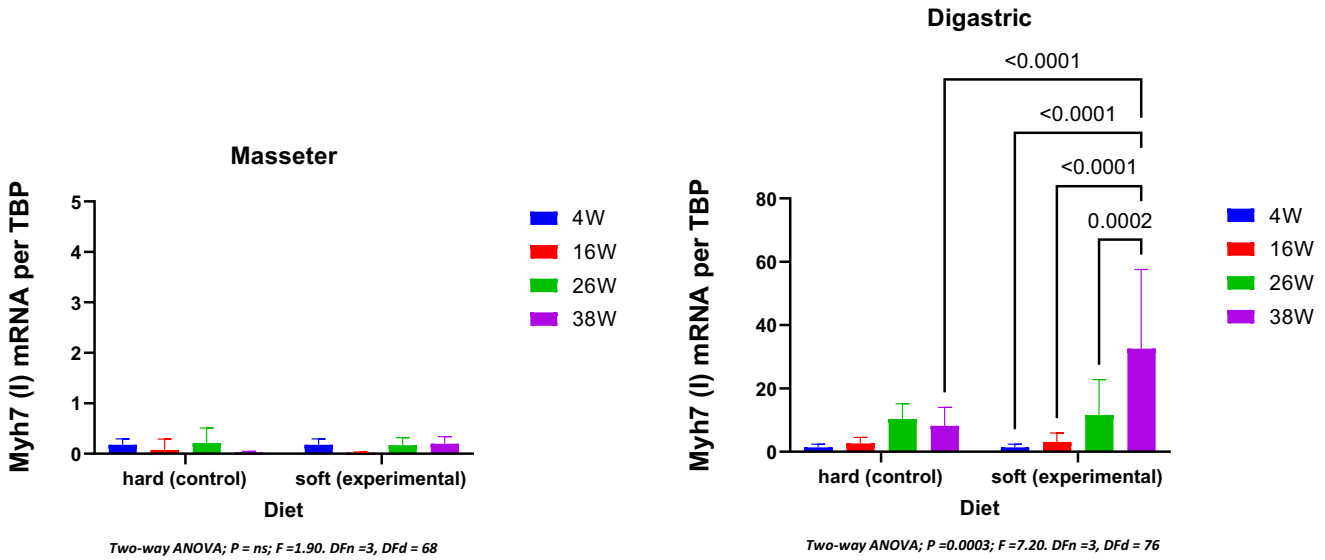

FIGURE 6 mRNA expression for *Myh7* at different time points for both experimental conditions for the masseter and digastric muscles. Only significant p values for post-hoc comparisons are shown. TBP, TATA binding protein; W, weeks of age.

3.3 | Myosin heavy-chain isoform expression related to masticatory function

When comparing rats subjected to hypofunction and normally fed rats, *Myh1* expression was greater in rats with hypofunction from 26 weeks of age onwards for the masseter muscles ($p < .0001$) but only at 38 weeks of age for the digastric muscles ($p < .0001$) (Figure 3). With regard to both *Myh2* and *Myh4* expression, rats with hypofunction showed a significantly decreased expression in the masseter ($p < .0001$, $p = .0456$) and digastric ($p = .0207$; $p = .0012$) muscles respectively by the age of 26 weeks (Figures 4 and 5). *Myh7* was not significantly expressed in either group in the masseter muscle, while in the digastric muscle it was significantly increased in rats with hypofunction at 38 weeks ($p < .0001$) (Figure 6).

4 | DISCUSSION

When rats are subjected to different masticatory functional demands, based on the present results, differences in the myosin expression profile in both the masseter and digastric muscles become mostly evident in adulthood (26 and 38 weeks in the present model), implying that perhaps maturation of the masticatory system and

the muscles is necessary in order for these differences to become evident.

Soft feeding seems to induce a significant reduction in the presence of fast-oxidative glycolytic fibres (Myh2; MyHC-IIa) in the masseter. The same results were found in previous studies[2,19] although these results were found in younger rats (8- and 12-week-old rats); soft feeding significantly reducing the relative frequency of type IIa fibres in the masseter muscle, while an increase in type I fibres was found in the digastric muscles. This could be explained by transformation into both glycolytic (*Myh1*; MyHC-IIX) and oxidative (*Myh7*; MyHC-I) fibre types within the digastric muscle since a significant increase in the frequency of glycolytic (*Myh1*; MyHC-IIx) and oxidative (*Myh7*; MyHC-I) fibres was found.

In contrary to the masseter muscle which is involved in the generation of powerful masticatory chewing forces, the digastric muscle is commonly involved in low-amplitude activities. Soft feeding, by reducing the occlusal load, could explain the increased expression of slow oxidative (*Myh7*; MyHC-I) fibres in the digastric muscle. Soft feeding has been described to change the chewing rates in masticatory cycles in rats[20] increasing them twofold.[15] In addition, electromyographic studies of the activity of masticatory muscles of rats fed a soft diet consisting of bread or pudding have shown a reduced activity in the masseter muscle[15] and the opposite has been shown in the digastric muscle,[19] this muscle being continuously active when animals were lapping their soft food.[15] Increased overall activity and increased amounts of low amplitude activities in the digastric muscles of 12-week-old rats fed a soft diet were described.[19] This continuous activity and the possible implied tongue motion which might help transport and process food could explain the increase in the digastric muscle of slow and fatigue-resistant fibres (*Myh7*; MyHC-I) when rats are fed a soft diet. When submitted to an increased activation and the necessity to counteract fatigue, increased levels of *Myh7* (MyHC-I) isoforms could be needed to adapt to new functional demands.

In the masseter muscle of rats fed a soft diet, a reduction in MyHC-IIa fibres could be explained by either transformation into a glycolytic fibre type or degeneration of oxidative fibres within the muscle. We are in favour of the first hypothesis since it is supported by a significant increase in the frequency of glycolytic (*Myh1*; MyHC-IIx) fibres within the masseter muscles of rats fed a soft diet.

In the masseter muscle, in rats fed either a hard or a soft diet, *Myh7* (MyHC-I) is very weakly expressed, which is in line with previous studies.[2,21] The weak presence of *Myh7* (MyHC-I) in the masseter muscle could be explained by its role in the power chewing stroke during which food is crushed. *Myh1* (MyHC-IIx) fibres being fast and fatigable, this type would predominate and be related to the generation of infrequent powerful contractions.[22] Electromyographic activity has also been shown to be reduced in the masseter muscles of rats fed a soft diet.[15]

A reduction in the masticatory strength and powerful muscle contractions required to chew soft food could explain the evolution of the MyHC isoform expression profile found in the masseter muscle. It has been previously shown that rats fed a soft diet present a decreased tetanic tension compared with those fed a normal hard diet.[23] Furthermore, in comparison with a normal diet, feeding rats a soft diet reduces the size of the fibres and also influences the distribution of fibre types present in the anterior deep masseter muscle.[5] Hypofunction, leading to reduced occlusal demands, might reduce the need for fast fatigue-resistant fibres in the masseter muscles which might explain the decrease in type IIa fibres and the concomitant increase in type IIx fibres.

Previous studies also described the same changes in the expression profile of different MyHC isoforms, although these changes could be seen earlier (9 days after weaning).[6,24] An increase in MyHC-IIb fibres in the masseter muscles of rats fed a soft diet has been observed in some studies,[2,6,19,24,25] contrasting with our results. Differences in the methodology, rat strain, locations of the dissected muscle parts and age could explain these differences. In addition, since the conversion of fibre types follows a strict order (MyHC-I ↔ MyHC-IIa ↔ MyHC IIx ↔ MyHC-IIb),[26] these variable results may also reflect the plasticity and dynamicity of myofibers. Furthermore, these variable results could be explained by the fact that our study limits itself to the quantification of the mRNA transcripts of the MyHC isoforms. It could be of additional interest to investigate whether a correlation with the protein expression of these MyHC isoforms is present.

Interestingly, in a murine model of Duchenne muscular dystrophy, it has been shown that at 12 months of age there is a reduction in type IIb MyHC isoforms and a corresponding increase in both type IIa and type IIx isoforms in masseter muscles.[27] If one looks earlier in the development of these mice, while the expression of MyHC-IIa seems to decrease and MyHC-IIb seems to increase in masseter muscles for the first few weeks after weaning, their expression levels are significantly lower in dystrophic than in healthy mice.[28] Instead, according to that study, MyHC-I which was hardly expressed in the masseter muscles of healthy mice, was seen to be strongly expressed several weeks after weaning in the dystrophic mice. These data may perhaps indicate there are some similarities between masticatory muscles subjected to hypofunction via a change in diet consistency, and those that undergo a degeneration and regeneration mechanism related to muscular dystrophy.

Our data suggest that long-term alteration of masticatory jaw function induced by a soft diet is associated with changes in the expression of different types of myosin and potentially fibre remodelling. Although these results cannot be directly extrapolated to human beings, the results may give insights into a better understanding of the functional changes that may occur in the masticatory muscles of humans and the resultant craniofacial morphology. In animal experiments, signals transmitted to the craniofacial bone and dento-skeletal tissues may have been reduced when masticatory muscles are submitted to hypofunctional demands, thus causing modifications in craniofacial growth[29,30] and changes in alveolar bone architecture during growth.[31] In humans, it has been described that masticatory muscle hypofunction can lead to the development of malocclusion[32] and a relationship has been described between craniofacial vertical patterns and the fibre-type composition of jaw-closing muscles.[33]

It is known that Individuals with a long-face pattern generate less jaw-closing activity[34] and demonstrate significantly weaker bite forces than normodivergent patients.[35–37] These differences with varying vertical facial characteristics can be reflected in the functional profile of the masseter muscle; individuals with short-face characteristics show an increased prevalence of type II fibres, while individuals with a long-face pattern have less type II and more type I fibres.[33] The masticatory function of patients with severe maxillofacial discrepancies can be improved with orthognathic surgery.[38,39] Surgical correction of a malocclusion and improvement in masticatory function[40] have been shown to induce changes in the MyHC composition[41] in masticatory muscles and a shift in the expression of MyHC isoforms from type I to type IIa, correlating with the number of teeth in occlusion.[40] In rats, tooth extraction has been shown to induce a change in function that impacts myofiber size and MyHC isoform expression in the anterior digastric muscle; however, these alterations can be mitigated by subsequent dental implant placement.[42] To date, bone-muscle cross-talk and interactions at a molecular and biochemical level have not been extensively studied in the masticatory system.[43] The bone-muscle interface may be of additional interest and provide insight into changes leading to alterations in the composition and maturation of the masticatory muscles as well as masticatory function and its influence on craniofacial bone morphology.

Limitations of the present study may include the possibility that other intrinsic factors, such as heredity, age, genetic background and hormones, may influence the fibre-type composition of skeletal muscles[44] and these were not investigated. Wistar rats were used in this study, and as MyHC isoforms are encoded by different genes, differences in genetic background between different rat strains might preclude the generalizability of these results. Moreover, as only male rats were used in this experiment, we were unable to analyse the influence of sex on the maturational changes taking place in jaw-opening and jaw-closing muscles. Finally, one cannot exclude that cognition may have been affected by soft feeding which may lead to modified masticatory muscle activity.[45]

Concerning the methodology, while immunohistochemistry quantifies the protein expression of MyHC, RT-qPCR provides mRNA transcripts of the MyHC isoforms. This method has been shown to be highly reliable with a good correlation between the mRNA and protein expression patterns.[11,12,46,47] One limitation of this method, however, is that it cannot be used to address the spatial variability in muscle-fibre composition; however, RT-qPCR overpasses the problem of hybrid muscle fibres that co-express more than one type of MyHC isoform simultaneously.[48,49]

5 | CONCLUSION

Hypofunction (soft diet) induces an increase in *Myh1* expression and a reduction in *Myh2* expression in masseter and digastric muscles, while in the latter an increase in *Myh7* expression was found in late adulthood. Together, these data suggest that long-term alteration of jaw function induced by the introduction of a soft diet is associated with changes in the expression of different types of myosins and potentially fibre remodelling.

AUTHOR CONTRIBUTIONS

Conceptualization and funding acquisition: S.K. Investigation: L.S., A.L. and A.A.-L. Validation, statistical analysis and resources: A.A.-L. Writing—original draft preparation and visualization: L.S. Writing—review and editing, supervision and project administration: G.S.A. and S.K. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/joor.13676.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, [LS], upon reasonable request.

ETHICAL APPROVAL

The present experiments were approved by the ethics committee for animal research (number GE/15/20A and number GE31) of the Canton of Geneva, Switzerland.

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