

**MASSETER MUSCLE MYOSIN 1C GENE EXPRESSION:
RELATIONSHIPS TO GROWTH FACTORS AND INFLUENCE ON FIBER-TYPE AND
SKELETAL GROWTH PATTERN**

A Thesis
Submitted to
The Temple University Graduate Board

In Partial Fulfillment
of the Requirements for the Degree
MASTER OF SCIENCE in ORAL BIOLOGY

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August, 2012

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ABSTRACT

The presence of dentofacial deformities in humans is prevalent, with distortions in jaw growth affecting about 20% of people worldwide. Least is known about the genetic etiology of malocclusions, so it is the purpose of this study to identify genetic factors which influence jaw growth and examine how their expression correlates with vertical and sagittal malocclusions. Myostatin (MSTN) is a negative growth regulator which functions to inhibit excessive growth of muscle. Mice in which this gene was absent exhibited increased muscle mass and altered skeletal form, indicating the role of genetic control on muscle mass and skeletal phenotype. IGF-1 is an anabolic growth factor which acts in coordination with growth hormone to promote myofiber regeneration and hypertrophy. A third gene of interest, myosin 1C is a class I myosin which functions to regulate glucose uptake via facilitated glucose transporter 4 (GLUT 4) in insulin and contraction stimulated pathways. Given its role in muscle metabolism in addition to its association to MYO1H, a paralogous protein which has been associated with Class III malocclusions, the goal of this study was to elucidate the possible role of MYO1C in mediating the metabolic effects of growth factors on fiber size and phenotype and subsequently skeletal form.

The aims of this study are as follows: quantify MYO1C expression in masseter muscle from individuals of different occlusal groups; compare MYO1C expression to myosin heavy chain gene expression and fiber percent occupancy by sagittal and vertical malocclusion classes; compare expression of MSTN and IGF-1 to MYO1C to evaluate if

a correlation exists; evaluate the expression of MYO1C and MYO1H to identify differences in proportions among malocclusion types.

Human masseter muscle samples were provided by oral surgeons at the University of Lille, France from subjects undergoing bilateral sagittal split osteotomy surgery for treatment of malocclusion. Muscle RNA was isolated with TRIzol™ reagent, digested with DNase I, re-isolated with RNAqueous® and quantified in 42 samples by triplicate assays of TaqMan® real time PCR using RNA-to-C_T™ 1-Step reagent and an Applied Biosystems Step One Plus instrument. A 25ng amount of skeletal muscle standard was selected as a reference calibrator and relative expression quantities of MYO1C were determined by the comparative threshold cycle (C_T) method. The relative quantity (RQ) of expressed RNA is calculated from the C_T value. Fiber type, area and percent occupancy had been determined previously for 39 of 42 masseter muscle samples used in this study. Expression of the genes for MyHC-I/β, IIA, IIX, perinatal (neonatal) and α (atrial) in another 39 of the 42 masseter muscle samples had also been previously quantified by RT-PCR for use in correlation analyses with MYO1C expression.

Based on the results collected, the final conclusions were drawn:

- MYO1C expression is greater in open and normal versus deep bites and Class III versus Class II malocclusions. The highest expression is seen with Class III open bites and the lowest with Class II normal malocclusions.
- Class II deep and normal bites showed high correlation between MYO1C expression and atrial and neonatal/atrial MHC gene expression, which require increased MYO1C

- for oxidative metabolism. They exhibited a negative correlation to type I MHC gene expression and percent occupancy, as deep bites have fewer type I fibers.
- Class III open bites had high correlation between MYO1C and neonatal MHC gene expression and low correlation to type II MHC gene expression due to increased percentage of high oxidative type I fibers in open bites and diminished type II fibers.
 - Correlations between MYO1C and hybrid I/II fiber percent occupancy was unpredictable by occlusal group due to transitional nature of fibers.
 - MYO1C expression is correlated to growth factor expression in Class III but not in Class II malocclusions, indicating its potential interactive role in masseter metabolism in the Class III group.
 - Class I myosins are highly expressed in Class III open bites.
 - Class II deep bites exhibited the lowest expression of MYO1H, indicating the masseters are less regulated by class I myosins.
 - MYO1H is closely linked with type II MHC gene expression, while MYO1C has a close association with types I and neonatal MHC gene expression.
 - An association exists between class I myosins and both type I and neonatal/atrial fiber percent occupancy.
 - A greater sample size of approximately 102 would permit an accurate test for significant differences in future studies.

ACKNOWLEDGEMENTS

First, I would like to thank Drs. Sciote and Horton for their guidance and support with this project. Throughout all the ups and downs we endured trying to get this project started, you have both been incredibly patient and very helpful. Dr. Sciote, thank you for being a great mentor and for getting me involved in this study. You truly have a passion for research and have been an invaluable resource of information. Dr. Horton, thank you for your countless hours of devotion to this study, I am not entirely sure I could have done it all without you. I greatly appreciate all of your assistance in completing and making this study a success.

Dr. Godel—Thank you for your leadership, and most importantly for your help in making this project possible. Without your tenacity in working out funding, there is no way I would have been able to complete my study. You clearly have a dedication to education and I have no doubt that you will help to make Temple's program even better than it is currently. We may not always express it, but your hard work has not gone unnoticed. I look forward to our graduation celebration and hope to karaoke with you.

Dr. Tuncay—Thank you for your unwavering dedication to Temple's Orthodontic program. You have made this program what it is today and have so much to be proud of. I have thoroughly enjoyed the time we've spent together and all I've learned from you, both about orthodontics and life. You have been a great fatherly figure to all of us, and while I know you're disapproving of my move to California, I do hope you'll come to visit.

Co-residents—To the Class of 2012, after two engagements, one baby and one wedding (and one to go), we finally made it! I am so grateful to have been in a class with five amazing women. Thank you for always supporting each other and not being swayed by the pressures of residency. I do hope that we will all keep in touch and while nothing will replace our convent-esque Tucson villa, maybe we can rent a crazy house for the AAO in the future. Classes of 2013 and 2014, thank you for being such wonderful co-residents. You have been fun, personable and quite frankly, a breath of fresh air. You are all extremely bright and I know you will succeed in all of your future endeavors. I wish you the best of luck with the remainder of your program!

Mom, Dad & Courtney—I'm not even sure where to begin. You have been so supportive of me attaining my goal of becoming an orthodontist, one that I declared at about the age of 10. I would never be where I am today without your steadfast love and support. Thank you for keeping me sane in times of stress, believing in me whenever I doubted myself and for loving me unconditionally throughout it all. I love you all and will miss you.

And last but certainly not least, Asher—you are my best friend, my love and my rock. I can't imagine going through the last six years of school without you, much less life in general. You have been my voice of reason and my silly better half, with whom I have the fortune of spending my future. Thank you for being so supportive of everything I do and pretending to be interested when I talk about dentistry all the time. I love you more than anything and look forward to a lifetime of happiness and adventures ahead of us.

Special Acknowledgements

S. Eugene Coben Endowed Scholarship—Without the generosity of Mrs. Rhoda Coben, this thesis project may not have been possible. Each year, Temple University's Department of Orthodontics awards scholarship awards to two students for research projects that focus on the basic science of growth and development in orthodontics. I would like to personally thank Mrs. Coben for awarding me this scholarship, as it has enabled me to complete this study and contribute to the advancement of the field of orthodontics. I sincerely appreciate your generosity and continued dedication to Temple's orthodontic program, which I know was an important part of your husband's life.

Patrick Hardigan—Thank you so much for your hard work in completing all of the statistical analyses for this thesis project. You were very helpful in explaining all of the statistical results, which was a great advantage given that I have no background in statistics. I especially appreciate that you were able to have everything completed in a timely manner, given our time restrictions toward the end of the study.

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CHAPTER 1

INTRODUCTION

The presence of dentofacial deformities in the human population is relatively prevalent, with distortions in jaw growth affecting about 20% of people worldwide (Wolford & Fields, 1999). These skeletal malocclusions are classified by differences in both sagittal and vertical facial dimensions. Vertical facial deformities (VFD), such as open and deep bites are very common in patients seeking orthodontic treatment and also difficult to treat. The majority of these distortions produce severe malocclusion of teeth, requiring interdisciplinary orthodontic and orthognathic surgical treatment to restore function and esthetics to the entire masticatory unit. It is estimated that approximately 1.8 million Americans have dentofacial deformities severe enough to require surgical intervention (Bailey et al., 1999). Given the prevalence and severity of these malocclusions in today's population, it is important to investigate the contributing factors which lead to the distortion in jaw growth.

The etiology of VFD is multifaceted, comprising behavioral, environmental and genetic elements. While much is known about the behavioral and environmental contributions to VFD, the genetic etiology of extreme facial patterns remains substantially unknown and requires further understanding of the basic physiologic, genetic and molecular mechanisms that control skeletal growth and adaptation (Rowlerson et al., 2005). The purpose of this study is to identify genetic factors which influence jaw growth and examine how their expression correlates with vertical facial deformities.

The importance of masticatory muscle function has been observed in anthropologic studies of Neanderthals, in which a low frequency of malocclusions was found in populations with primitive living conditions. In contrast, as diets have become more refined, the demand for heavy masticatory forces has decreased and the prevalence of malocclusions has increased among contemporary individuals. These trends indicate that there is indeed a relationship between form and function in the masticatory system. Recent work has shown that altered masticatory functioning has significant effects on the face and neurocranium (Havarti & Weaver, 2006), as bone remodels to reach the shape that can best withstand the mechanical loads applied. Similar results were seen in mice studies where reduced masticatory function, as a result of a soft diet, led to lower bone apposition in the angle of the mandible, which consequently increased anterior facial height in the mice (Kiliaridis, 1985).

Because the relationship between muscle activity and jaw growth is so complex, an important aspect to consider is the intrinsic composition of muscle in terms of its fiber-type composition. Rowleson et al. (2005) showed that the fiber type composition between human limb and masseter muscles is very different. Limb muscles have predominantly type II fibers (~60%), few hybrid I/II fibers and no neonatal/atrial types. In contrast, masseter muscles have on average smaller fiber diameters than limb, especially for type II, a predominance of type I fibers, proportionately more hybrid I/II fibers and neonatal/atrial hybrid types. When muscle samples from open and deep bite patients were examined, deep bite subjects had a substantial increase in type II fiber occupancy and open bite subjects a decreased occupancy in comparison to normal

subjects. Given the variation in fiber-type composition between different occlusal groups, the underlying genetic expression controlling this composition must be evaluated.

Two genes, myostatin (MSTN or GDF-8) and insulin-like growth factor 1 (IGF1), have been identified as potent regulators of muscle fiber-type variations. MSTN is a negative growth regulator which functions to inhibit excessive growth of muscle in order to maintain an overall balance of this tissue relative to adipose tissue. In two studies evaluating the differences between myostatin-deficient and wild-type mice, Vecchione et al. (2007; 2010) found that myostatin-deficient mice not only had significantly greater body and muscle weight, but also brachycephalic crania with smaller cranial dimensions compared to wild-type controls. IGF1, on the other hand, is a positive regulator of muscle fiber-type variations. In several studies, the sustained local overexpression of IGF1 was shown to promote myofiber regeneration and hypertrophy and to increase levels of myogenic regulatory factors and contractile protein mRNAs (Musaro et al., 2001; Coleman et al., 1995; Fiatarone et al., 1999).

Both IGF-1 and MSTN have a clear role in regulating muscle growth and repair, and therefore have an indirect effect on skeletal development. In a first report of a genetic variation associated with sagittal facial dimension, another gene of interest, myosin 1H (MYO1H) has been linked to Class III malocclusion in a Caucasian population (Tassopoulou et al., 2011; 2012). Myosin 1H is a type I myosin, which are single-headed monomers involved in regulation of membrane dynamics, intracellular vesicle transport, inner ear auditory function and transcriptional motor function. Among the class I myosins, MYO1C and MYO1H are vertebrate-specific paralogs (Hofman et al., 2009),

with MYO1C being the more prominent species. The MYO1C protein regulates glucose uptake via facilitated glucose transporter 4 (GLUT 4) in mouse skeletal muscle by acting as a motor for movement of GLUT4-stored vesicles to plasma membranes in both insulin and contraction stimulated pathways (Toyoda et al., 2010). Expression of MYO1C was found to increase in association with increased muscle contraction, insulin-stimulated glucose uptake and increased oxidative capacity.

As a major player in the control of glucose uptake into muscle, MYO1C may mediate the metabolic effects of growth factors on fiber size and phenotype. Therefore, it is important to evaluate the expression of MYO1C in muscle from different occlusal groups for correlation to fiber type and mean percent occupancy. This study aims to execute that correlation analysis in addition to comparing MYO1C expression to that of MSTN, IGF1 and MYO1H.

CHAPTER 2

REVIEW OF THE LITERATURE

2.1 The Evolution and Etiology of Malocclusion

Considering the prevalence of malocclusion in today's population, it may surprise many that malocclusion is a relatively recent phenomenon which appeared after transition to industrialized civilization. The importance of masticatory muscle function has been observed in anthropologic studies, in which a low frequency of malocclusions was found in populations with primitive living conditions. The increase in malocclusions was thought to be either a result of the premature loss of deciduous teeth due to caries or an effect of an alteration in consistency of the diet, reducing masticatory functional requirements (Kiliaridis et al., 1995). Studies of the Inuit (i.e. Eskimo) skull have also exhibited a correlation between masticatory function and craniofacial form. The Inuit skull is characterized by a large mandible, larger muscle attachments, and palatal and mandibular tori. According to Collins' "hard chewing hypothesis", the distinctive shape of the Inuit skull is related to vigorous chewing; the skulls adapted to produce and dissipate large vertical and biting forces. The masseter muscles are also positioned more anteriorly, which may help generate larger forces (Hylander, 1977).

Similarly, Neanderthal facial configuration seemingly adapted to use anterior teeth for repeated force production in many functions. Examples of such functions include: mastication of abrasive foods, and clamping of non-dietary hard objects such as holding small pieces for construction or slats of wood. According to Spencer & Demes (1993), Neanderthals were well designed for efficient incisal use; this increased

efficiency arose by virtue of an anterior migration of the masticatory muscles and a diminution of the distance between the incisors and temporomandibular joint (TMJ). The facial skeleton was modified to increase resistance to masticatory forces and maximize function for their everyday tasks. Moreover, craniofacial form was optimized for the production of high forces on the anterior dentition and the retention of some critical occlusal area on the post-canine tooth row.

Findings that correlate form and function are not limited to primitive populations. As time has passed and human diets have become more refined, the demand for heavy masticatory forces has been reduced and the prevalence of malocclusions has increased among contemporary individuals. The resultant decrease in condylar growth, with a tendency toward a posterior rotation of the mandible could contribute to the increased frequency of Class II malocclusion in modern man (Varella, 1992). In contrast, Kiliaridis (1995) found that humans with an increased level of masticatory muscle activity due to bruxism have a lower incidence of malocclusions and are characterized by a short lower face, a smaller intermaxillary angle, and a smaller gonial angle than adult norms. In another study of craniofacial morphology in men and women with advanced attrition, extensive occlusal wear due to bruxism was also positively correlated to increased bite force, indicating higher than normal masseter muscle functional activity. The sample of men and women with advanced occlusal wear had a low frequency of orthodontic problems in the transverse, vertical and arch length dimensions when compared with epidemiologic studies done on healthy Swedish adults (Kiliaridis et al., 1995; Ingervall et al., 1978; Mohlin, 1982). Thus, masticatory muscle activity is a key environmental influence on the development of skeletal malocclusion.

2.2 Muscular Influences on Bone Remodeling

Recent work has shown that altered masticatory functioning has significant effects on the face and neurocranium (Havarti & Weaver, 2006). Masticatory muscles are inserted into the mandible by means of their aponeuroses through the periosteum or tendons, and through them they exert tensile forces on the bone (Dorfl, 1980; Harvold, 1985). Bone remodels to reach the shape that can best withstand the mechanical loads applied. Thus, prolonged application of a bending force on a long bone stimulates bone apposition on the surface that becomes more concave and bone resorption on the surface that becomes more convex. Conversely, the reduction or absence of mechanical stress on regularly load-bearing bone induces a general remodeling and reduction of bone mass resembling a disease osteoporosis (Kiliaridis, 1995). Previous studies investigating the impact of a change in diet consistency in mice have helped to confirm the environmental influence of masticatory muscle activity on bone growth (Beecher & Corruccini, 1981; Bouvier & Hylander, 1984; Kiliaridis, 1986; McFadden et al., 1986; Watt & Williams, 1951; Yamada & Kimmel, 1991). Reduced functional demands on the masticatory muscles, induced by feeding the animals a soft diet, promotes the remodeling of the bony structures of the upper viscerocranium and the mandible. Changes are also noted in the size and dimensions of the alveolar process, such as the width, height, and thickness of alveolar bone, resulting in a smaller cross-sectional area of the bones (Kiliaridis, 1985). The reduced masticatory function led to lower bone apposition in the angle of the mandible, which consequently increased anterior facial height in the mice.

In addition to craniofacial differences noted, Kiliaridis and coworkers also report that the masticatory muscle fiber type composition and size changes with the alteration in diet. In the anterior deep masseter, they found that the percentage of type IIA fibers is decreased, and the percentage of type IIB fibers is increased in the soft-diet group when compared to a normal-diet group (Kiliaridis et al., 1988). This suggests that in the soft-diet group, less exercise produced lower oxidative potential and more sensitivity to fatigue than in the normal diet group, in which the masticatory demand was higher. From these studies, we can infer that in mice fed a soft diet, the elevator muscles do not encounter the same functional demands as in rats fed a normal diet so the masticatory muscles develop differently, followed by changes in the craniofacial growth pattern (Kiliaridis et al., 1985; Engstrom et al., 1986).

Nonhuman primates have also been studied by placing one group on a soft artificial diet and comparing them to another group on a natural tough consistency diet. This experimental approach, using a natural, non-invasive mechanism, showed a statistically significant association between occlusal abnormalities and maintenance during growth on a soft diet that is relevant to humans. The squirrel monkeys on the altered diet developed long, narrow maxillary arches with increased overjet, impacted and malerupted premolars, and crowded incisors (Corruccini & Beecher, 1982). Reduced masticatory function caused decreased transversal dimensions of the dental arch breadth, which could explain the greater frequency of space-related malocclusions. Because these changes occurred within one generation and over short periods of time, they are most likely the result of changes in gene expression by bone, cartilage and muscle rather than from genetic variation in offspring (Sciote, 2012).

Since many muscular disorders result in weak muscles, a closer view of deviations occurring in the craniofacial growth of affected patients may give better insight into the influence of muscle function on dentofacial growth and morphology. How variation in muscle activity affects craniofacial growth may be best demonstrated in children with Duchenne Muscular Dystrophy (DMD) who develop severe open bite malocclusions due to decreasing masticatory muscle forces transmitted to the face as the condition progresses (Eckardt & Harzer, 1996). The progression of the disease is dramatic from the age of 7-8 years, and this is about the time when the activity of the jaw muscles greatly diminishes (Kiliaridis & Catsaros, 1998). The facial morphology of patients with DMD has been shown to be retrognathic, with sagittal underdevelopment of the cranial, maxillary, and mandibular base and relatively large lower facial height (Kiliaridis & Catsaros, 1998). The sagittal underdevelopment and transversal overdevelopment of dental and skeletal structures results from the lack of activity of the masticatory muscles and increased tonus of the perioral muscles. These results suggest a relationship between the function of the masticatory muscles and the shape of the facial bone complex.

2.3 Fiber Type Composition in Skeletal Muscle

Because the relationship between muscle activity and jaw growth is so complex, an important aspect to consider is the intrinsic composition of muscle in terms of its fiber-type composition. Myosins are large ATP-dependent motor proteins that exhibit actin binding and ATP hydrolysis for functions in cellular motility and force transduction

in muscle contraction. In skeletal muscle, Class I and II myosins, respectively, perform these functions. Skeletal muscle fiber phenotypes have been classified by different myofibrillar ATPase activities of the class II myosins that are responsible for muscle contraction. The typical classification system upon which skeletal muscle fiber types are based includes three main groups: type I, IIA and IIB. The different histochemical staining properties of the ATPase moieties of the myosin heavy chain (MyHC) protein isoforms are used to identify these three main fiber types. Physiologic studies have shown that type I fibers are fatigue resistant and have slow shortening velocities, whereas type II fibers are relatively fatigable and have more rapid shortening velocities. For example, postural muscles are composed mostly of type I fibers, whereas muscles used in rapid locomotion have higher proportions of type II fibers. In actuality, many skeletal muscles are composed of a variety of fiber types with different functional and histological characteristics. To characterize the atypical fiber-type composition in human masticatory muscles, Sciote et al. (1994) developed a classification system of four fiber phenotypes: type I, type II that includes type IIA and IIX, type I/II hybrids that express both type I and II MyHC proteins, and type neonatal/atrial (N/A) that express a developmental isoform and the cardiac alpha myosin (MyHC- α) isoform.

Rowlerson et al. (2005) showed that the fiber type composition between limb and masseter muscles is very different; the limb muscles had approximately 40% type I and 60% type II fibers, but few I/II hybrid, while type I fibers occupied approximately half and type II fibers only 15% of the tissue volume (i.e. mean percent occupancy; MPO) of masseter muscle. The masseter type II fibers were also smaller in size, therefore producing substantially less force relative to those in limb muscles. Interestingly, when

masseter samples from normal vertical dimension patients were compared to those from open and deep bite patients, there were significant differences in MPO among the fiber types. The size and occupancy of type I fibers differed little in open bite subjects, but the occupancy of type II fibers drops to 8% (Rowlerson et al., 2005). Conversely, deep bite subjects showed decreased occupancy of type I fibers and a substantial increase in type II fiber occupancy. This study demonstrated that vertical bite characteristics vary with the fiber-type composition of masseter muscle, and that increased type II fiber MPO appears to be correlated with a decrease in vertical facial height.

In a follow-up study, the association between masseter phenotype and mandibular asymmetry was examined by comparing left and right side samples of muscle from orthognathic surgery patients (Raoul et al. 2011). Type II fiber occupancy was reported to be significantly increased on the same side as the deviation, i.e. on the ‘short side’ of the mandible. Collectively, these results indicate that decreased vertical lower facial height and mandibular ramus shortening is significantly linked to type II fiber percentage occupancy. Since type II fibers in larger motor units are normally recruited at higher contraction strengths (Milner-Brown et al., 1973), the subsequent increased force in these muscles may contribute to the altered mandibular form. This hypothesis coincides with data from Duchenne Muscular Dystrophy subjects, where the opposite condition, masticatory muscle weakness, progressively causes skeletal open bite malocclusions. So while it is known that masseter volume does not correlate with asymmetry (Kwon et al., 2007), the composition of the muscle in terms of the fiber phenotypes is associated with craniofacial form.

2.4 Genetic Control of Skeletal Muscle Metabolism

The mechanisms by which non-syndromic deviations in craniofacial morphology develop are complex and not fully understood, but ongoing studies for heritable influences on bone length and muscle strength phenotypes have revealed a number of important associations. The Genetic Investigation of Anthropometric Traits (GIANT) consortium has identified 180 variants which affect human height (Lango et al., 2010). Additionally, the Human Gene Map for Performance and Health-Related phenotypes has summarized genes or genetic loci which affect fiber types and muscle strength during adolescence and young childhood (Bray et al. 2008). Within this complex of genetic interactions, two genes, myostatin (MSTN or GDF-8) and insulin-like growth factor 1 (IGF1), have been identified as potent regulators of muscle fiber-type variations. Myostatin is a negative regulator of muscle growth; in normal physiology, it functions to inhibit excessive growth of muscle in order to maintain an overall balance of this tissue relative to adipose tissue. The gene is highly conserved across species, and a spontaneous gene mutation associated with increased muscle mass has been identified in humans (Schuelke et al., 2004). When MSTN activity is blocked, more cells are committed to myoblast and myotube formation that results in significantly elevated numbers of muscle fibers in adult muscle (Welle, 2009). Average fiber diameter is also increased, demonstrating that both hypertrophy and hyperplasia are responsible for increased muscle mass. Fiber type distributions are also altered; knockout mice have decreased numbers of type I fibers and increased numbers of type II fibers, especially the type IIB, glycolytic

fast-contracting fiber (Salerno et al., 2004; Girgenrath et al., 2005). In a study of possible effects of MSTN on insulin-like growth factors, Kocamis et al. (2002) reported that IGF2 but not IGF1 expression was significantly higher in soleus muscle of myostatin knockout mice. Also, mRNA levels for the IGF receptor (IGF-R1) were significantly higher in heart and lower in pectoralis muscle of control than in knockout mice indicating that interactions between these negative and positive regulatory molecules are complex and possibly tissue and muscle specific in nature.

2.4.1 Myostatin as a Negative Regulator of Growth

The myostatin knockout mouse is of interest to craniofacial biology investigation because its masticatory muscles have been demonstrated to be significantly larger. In two reports of the differences between myostatin-deficient and wild-type mice, Vecchione et al. (2007; 2010) found that myostatin-deficient mice not only had significantly greater body and muscle weight, but also brachycephalic crania with smaller cranial dimensions, foreshortened midfaces and a lengthened mandibles, which were shorter in vertical dimension, compared to wild-type controls. These results are similar to those reported by Byron et al. (2008) who showed that adult myostatin-deficient mice had significantly shortened and compressed (in an anterior-posterior dimension) temporal bones and a smaller squamosal suture bevel compared to wild-type controls. Significant associations noted between masseter muscle weight and several craniofacial dimensions suggest that hypermuscularity and increased bite force probably played a significant role in producing the altered craniofacial morphology. With the loss of myostatin, the delicate signaling

pathways between cell proliferation, protein synthesis, and tissue interactions and skeletal growth are probably thrown out of balance in early developmental stages. These findings emphasize the important role that masticatory muscle function plays in the development of the cranial vault, maxilla and mandible.

2.4.2 IGF-1 as a Positive Regulator of Growth

The other gene of interest, IGF1 has been identified as a positive regulator of muscle fiber-type variations that, unlike MSTN, promotes increased muscle growth. IGF1 is a central component of the growth hormone (GH)/IGF1 axis in the regulation of muscle growth via metabolic and anabolic actions (Philippou et al., 2007). Circulating IGF1 is mostly derived from the liver, but also from skeletal muscle and adipose tissue (Naranjo et al., 2002), and acts in an endocrine manner under the control of circulating levels of GH. In addition to being the target of GH, skeletal muscle also produces its own IGF1 that acts in a paracrine fashion to affect growth. The circulating or locally produced IGF1 in target tissues mediates the muscle growth-promoting actions of GH. In several studies, the sustained local overexpression of IGF1 was shown to promote myofiber regeneration and hypertrophy and to increase levels of myogenic regulatory factors and contractile protein message expression (Musaro et al., 2001; Coleman et al., 1995; Fiatarone et al., 1999). Interestingly, increases in serum IGF1 with exogenous administration of GH or IGF1 does not appear to stimulate myofiber hypertrophy in the absence of mechanical loading (Bamman et al., 1998). Therefore, it is possible that

regulation of local production of IGF1 isoforms may be tissue-specific concerning the molecular mechanisms by which synthesis is modulated.

IGF1 is a main systemic regulator of tissue mass during postnatal growth and in addition to its role in muscle hypertrophy, it contributes heavily to repair in post-mitotic skeletal muscle cells following local injury. Cellular processes of myofiber regeneration and hypertrophy are enabled by the activation, proliferation and subsequent differentiation of quiescent mononuclear muscle stem cells. Previous studies have shown that these processes appear to be modulated by the autocrine and/or paracrine-produced IGF1 (Adams, 2002a, 2002b). One particular study examined the expression of IGF1 splice variants in muscle tissue following both mechanical damage imposed by electrical stimulation of the stretched muscle and damage caused by injection with bupivacaine. Hill & Goldspink (2003) found that IGF1 is differentially spliced in response to local muscle overload and damage, and that the autocrine splice variant mechano growth factor (MGF) initiates muscle satellite cell activation. This local splice variant provides extra, undamaged nuclei required for muscle fiber repair with some upregulation of protein synthesis. Thereafter, splicing switches to primarily produce systemic the IGF-IEa variant in the liver, which upregulates protein synthesis over a somewhat longer time scale. Overexpression of IGF1 splice variants, whether induced by hormone or local muscle damage, is extended beyond that required for local repair and results in muscle hypertrophy.

2.4.3 Association of MYO1H with Mandibular Prognathism

Both IGF-1 and MSTN have a clear role in regulating muscle growth and repair, and therefore have an indirect effect on skeletal development. In a first report of a genetic variation associated with sagittal facial dimension, another gene of interest, myosin 1H (MYO1H) has been linked to Class III malocclusion in a Caucasian population (Tassopoulou et al., 2011; 2012). Researchers detected a single nucleotide polymorphism (SNP) within the MYO1H gene that is over-represented in a population of European ancestry with prognathism. Although MYO1H is not itself a growth factor, evidence of this genetic polymorphism indicates a potential clinically significant relationship between growth and function of muscular and skeletal components of the face. Unlike the class II myosins, which are responsible for muscle contraction and are the basis for histochemical classification of skeletal muscle fiber types, class I is an unconventional myosin group of single-headed monomers involved in regulation of membrane dynamics, intracellular vesicle transport, inner ear auditory function and transcriptional motor function. Eight class I myosin genes in humans, designated MYO1A to MYO1H, act as tension-sensors that respond to load changes by altering their ATPase and mechanical properties (Laakso et al., 2008).

2.4.4 Role of MYO1C in Glucose Metabolism

Among the class I myosins, MYO1C and MYO1H are vertebrate-specific paralogs (Hofman et al., 2009), with MYO1C being the more prominent species. The

MYO1C protein regulates glucose uptake via facilitated glucose transporter 4 (GLUT 4) in mouse skeletal muscle by acting as a motor for movement of GLUT4-stored vesicles to plasma membranes in both insulin and contraction stimulated pathways (Toyoda et al., 2010). Specifically, expression of MYO1C was found to increase in association with increased muscle contraction, insulin-stimulated glucose uptake and increased oxidative capacity. Likewise, MYO1C decreases in sedentary mice and insulin resistant tissues. Another function has been shown for the variant 3 isoform of MYO1C, which is found in nuclei and called nuclear myosin I (Pestic-Dragovich et al., 2000), and acts as a molecular motor in transcriptional complexes (Percipalle et al., 2006).

2.5 Relationship of Class I Myosins to Fiber-Type & Skeletal Growth Pattern

The association of MYO1H with mandibular prognathism suggests there may be an important link between functions of class I myosins and musculoskeletal development for sagittal jaw deformation. Although MYO1H has yet to be assigned a specific cellular function, it may participate as a motor protein in glucose transport and transcription like MYO1C. Preliminary results from quantitative real time polymerase chain reaction assays indicate that MYO1H expression is low in muscle and particularly lower in masseter than in limb muscle (Gray et al., 2012). An analysis using an Agilent 4x44 whole human genome microarray to compare gene expression between a pool of limb muscle RNA and masseter muscle RNA from open and deep bite subjects did not include probes to MYO1H, but data from this same microarray shows that MYO1C expression is detected at high levels in muscle tissues (Horton, et al., unpublished results). In that

array, the average expression of MYO1C is -2.07 fold different between deep bite and open bite specimens. Among ten glucose transporter genes on this same array, the highest expression levels were seen for GLUT4, which was also down-regulated -2.79 fold in the masseter from the deep bite subject. As a major player in the control of glucose uptake into muscle, MYO1C may mediate the metabolic effects of growth factors on fiber size and phenotype. In another microarray analysis, Chen et al., (2010) revealed that MSTN up-regulates several genes involved in regulation of glucose metabolism including GLUT4. By promoting glucose uptake and consumption via glycolysis in skeletal muscle, MSTN may elicit effects on fibers through energy metabolism. Likewise, IGF1 as a promoter of growth also affects metabolism in skeletal muscle.

Because the metabolic profiles of type I and II fibers are unique, expression of MYO1C in the two fiber types may be different. Type II fibers are more sensitive to glucose, because they rely solely on glycolysis to make ATP, while type I fibers undergo oxidative metabolism, producing a higher quantity of ATP. To further elucidate the metabolic differences between fiber types, Sciote et al. (2012) examined the ratio of growth factors IGF-1:MSTN in type I and II fibers of both males and females. In females, type I mean fiber areas increased as the ratio of IGF-I to MSTN increased ($R^2 = .405$) (Figure 1), and in males type II mean fiber areas decreased as the ratio of IGF-I to MSTN increased ($R^2 = .413$) (Figure 2). These plots suggest that the two fiber types are differentially affected by proportionate quantities of growth factors. Although this is a promising data trend, larger subject numbers are needed to explore these relationships, as the data used are missing stratification by age and malocclusion class. While one would expect the mean fiber area to increase with the ratio of IGF-1:MSTN due to the anabolic

role of IGF-1, type II fibers appear to respond differently to growth factor regulation. As a mediator in glucose metabolism, MYO1C may also show a variable expression pattern in type II fibers. Considering their prevalence in deep versus open bite masseter muscles, MYO1C may be present at different levels in these patients.

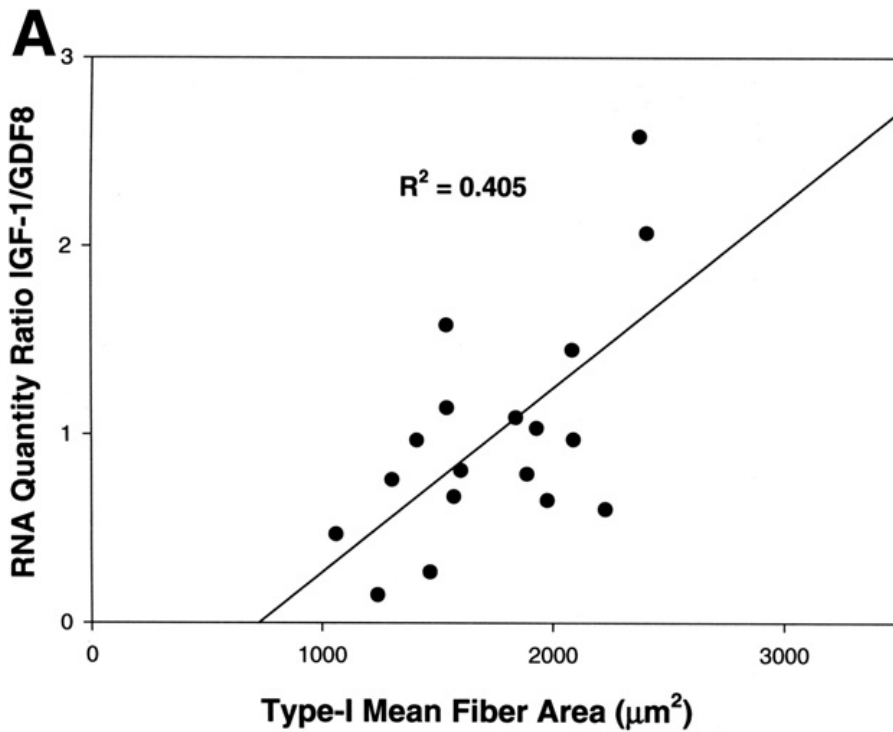


Figure 1- Linear regression analysis of relative quantities of IGF-1 and GDF-8 RNA ratios and type I mean fiber area values for 18 female masseter muscles (Sciote et al., 2012)

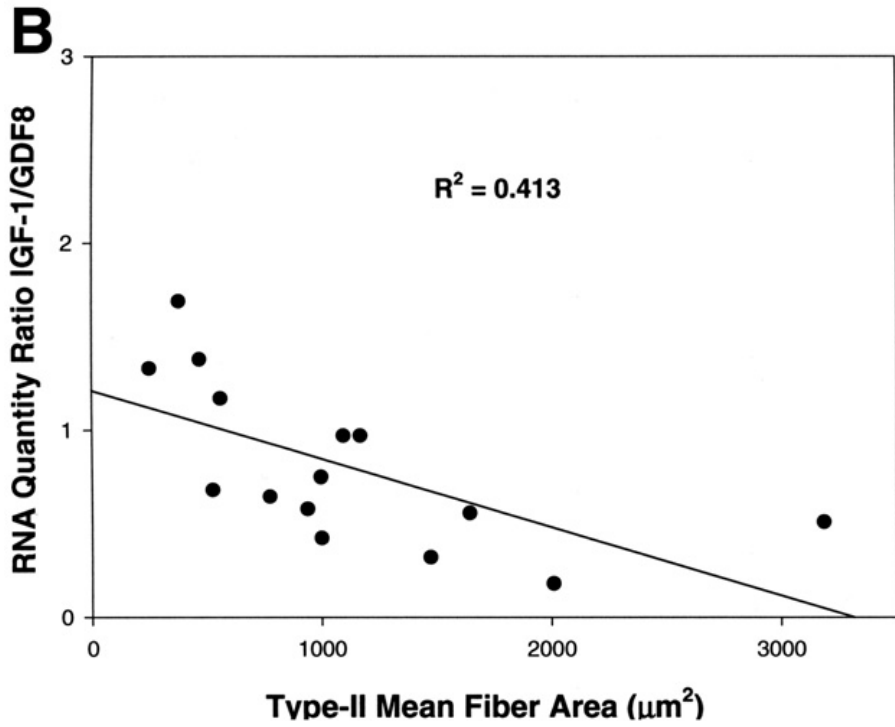


Figure 2- Linear regression analysis of relative quantities of IGF-1 and GDF-8 RNA ratios and type II mean fiber area values for 15 male masseter muscles (Sciote et al., 2012)

CHAPTER 3

AIMS OF THE INVESTIGATION

This study has four specific aims. First, MYO1C gene expression will be quantified in masseter muscle from individuals of different occlusal groups. Second, data for MyHC gene expression and mean percent occupancy of fiber types for each of the samples will be analyzed for correlation to MYO1C expression by both sagittal and vertical malocclusion classes. Third, expression of MSTN and IGF-1 will be compared to MYO1C to evaluate if a correlation exists. Fourth, the expression of MYO1C and MYO1H will be compared to identify differences in proportions among malocclusion types.

CHAPTER 4

MATERIALS & METHODS

4.1 The Sample

Procedures for acquisition of masseter muscle samples have been established (Rowlerson et al., 2005). Specimens were previously obtained from subjects undergoing orthognathic surgery for treatment of malocclusion at the University of Lille, France (see Appendix A). A summary of numbers of subjects and characteristics is shown in Table I.

Table 1. Orthognathic Surgery Subjects

<u>Gender</u>	<u>Subjects (n)</u>	<u>Mean Age</u>	Occlusal Types			
			<u>Vertical Dimension</u>	(n)	<u>Sagittal Dimension</u>	(n)
Females	24	20.21	Normal Bite	8	Class I	1
			Open Bite	13	Class II	15
			Deep Bite	3	Class III	8
Males	19	20.11	Normal Bite	5	Class I	0
			Open Bite	6	Class II	10
			Deep Bite	8	Class III	9

Masseter samples were taken 1.5 cm from the lowest point of the mandible's angle during bilateral sagittal split osteotomy procedures and processed for histologic analysis. Consent for subject participation was obtained according to research ethics committee regulations at the University of Lille and the Institutional Review Boards of the University of Pittsburgh and Temple University. Malocclusion classification of

subjects was based upon jaw dimension repositioning required in the surgical treatment plan.

RNA from human forearm extensor, trapezius, quadriceps and triceps/deltoid limb muscles had been obtained from orthopedic surgeries of earlier studies with approval (King's College, London and the University of Pittsburgh) and banked for comparative use with masseter muscle studies (Appendix D).

4.2 RNA Isolation & Real Time PCR

Muscle RNA was isolated with TRIzol™ reagent (Invitrogen, Carlsbad, CA), digested with DNase I, re-isolated with RNAqueous® and quantified by absorbance at A₂₆₀ as described previously (Horton, 2008). MYO1C RNA was quantified in 42 samples by triplicate assays of TaqMan® (Applied Biosystems, Foster City, CA) real time PCR using RNA-to-C_T™ 1-Step reagent and an Applied Biosystems Step One Plus instrument. Reactions included Applied Biosystems specific primer-probe sets for MYO1C (Hs00300761_m1) and for the endogenous control gene hypoxanthine phosphoribosyltransferase-1 (HPRT1; Hs01003267_m1). Primer-probe sets are designed to span exon junctions to avoid detection of any genomic DNA. Commercial human skeletal muscle RNA (Ambion) and human thymus RNA were used as a positive tissue controls and reference standards for comparison with the biopsied muscle tissues. A standard set of conditions for reverse transcription and amplification were used in all assays (Table II).

Table 2 Standard conditions for RT-PCR

<u>Step</u>	<u>Temperature (°C)</u>	<u>Time</u>	<u>Cycles</u>
Reverse Transcription	48	15 min	Hold
Enzyme Activation	95	10 min	Hold
Denaturation	95	15 sec	40
Anneal/Extend	60	1 min	

Initial RT-PCR assays used serial dilutions of skeletal muscle RNA to show that amplification efficiencies of HPRT1 endogenous control and MYO1C were within 10% of one another (Figure 3). Expression of MYO1C in the thymus was lower in comparison to skeletal and masseter muscle, which showed similar expression. All limb data can be seen in Appendix D.

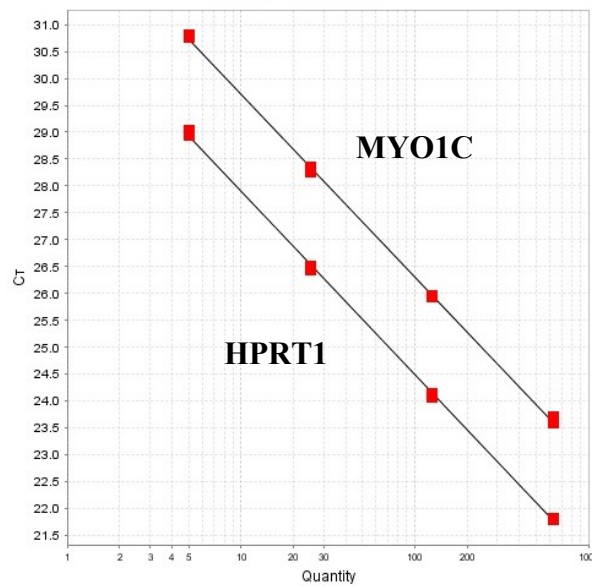


Figure 3. Comparison of RT-PCR amplification plots of MYO1C and HPRT1 using standard quantities of 5ng, 25ng, 125ng and 625ng a commercial human skeletal muscle RNA.

After these initial tests to establish assay conditions, a 25ng amount of skeletal muscle standard was selected as a reference calibrator and relative expression quantities were determined by the comparative threshold cycle (C_T) method ($\Delta\Delta C_T$) of Livak and Schmittgen, 2001. This method measures fold-difference between normalized amounts of target in test samples and in an internal reference when reference and target genes are amplified at approximately the same efficiency. The relative quantity (RQ) of expressed RNA is calculated using the equation:

$$RQ = 2^{-\Delta\Delta CT}$$

$$2^{-\Delta\Delta CT} = 2^{-[\Delta CT (\text{Muscle Sample}) - \Delta CT (\text{Reference})]}$$

$$\Delta C_T (\text{Muscle Sample}) = C_T (\text{MYO1C in Biopsy Muscle}) - C_T (\text{HPRT1 in Biopsy Muscle})$$

$$\Delta C_T (\text{Reference}) = C_T (\text{MYO1C in Muscle Standard}) - C_T (\text{HPRT1 in Muscle Standard})$$

Thus, measurements of MYO1C are normalized to HPRT1, and RQ is determined by comparing the normalized MYO1C quantity in each sample of masseter or limb muscle to the normalized MYO1C quantity in the reference sample of skeletal muscle RNA.

4.3 Analysis of Fiber type Properties

Fiber type, area and a percent occupancy (the percent of muscle area occupied by a fiber type) had been determined previously for 39 of 42 masseter muscle samples used in this study (Rowlerson et al., 2005). Specimens had been snap-frozen, cryosectioned serially at 10 μm and the sections mounted on glass microscope slides for

immunohistochemical staining by an indirect immunoperoxidase method. The antibodies used here were specific for myosin heavy chain (MyHC) isoforms: type-I (BA-F8), all type-II (MY-32), type-IIA only (SC-71), neonatal (a polyclonal antibody prepared by Dr. Anthea Rowleron) and α -cardiac (MAS 366). Immunostaining of masseter muscle with this set of antibodies permitted identification of 8 fiber types, which were organized into 4 groups as follows: type-I, containing only type I MyHC; type-II, containing only type IIA and/or IIX MyHC; type I/II hybrid fibers, containing both type I and II isoforms; and type neonatal/atrial fibers, containing neonatal or α -cardiac MyHC in combination with type-I or type-II isoforms.

The cross-sectional area of identified fibers was measured with image-analysis software by displaying each digital image and tracing its outer border with a VIDS-V image-analysis system (Ai, Cambridge, United Kingdom) linked to a Nikon Labophot microscope (Nikon, Tokyo, Japan). Fibers with adequate staining and morphology were included and damaged fibers rejected for analysis. Fiber areas were used to calculate the percent occupancy for each of the 4 fiber groups. For the present study, mean percent occupancy values for the fiber types were calculated and this data used to analyze correlations to MYO1C expression by both sagittal and vertical malocclusion classes.

Expression of genes for MyHC-I/ β , IIA, IIX, perinatal (neonatal) and α (atrial), in another 39 of the 42 masseter muscles samples had been previously quantified by RT-PCR according to the method of Horton et al. (2008). A sum of the expression values for the 5 different MyHC genes was calculated for each specimen, and a percent of that total

was determined for each MyHC type for use in correlation analyses with MYO1C expression.

4.4 Statistical Analyses

Descriptive statistics including mean and standard deviation were calculated to analyze the data set by sex, gender, separate vertical and sagittal malocclusion groups, and combined vertical and sagittal malocclusion groups.

4.4.1 ANOVA Analyses

Using a 2-way ANOVA with interaction, a model was created consisting of the variables: sex, age, vertical malocclusion groups (deep, normal, open), sagittal malocclusion groups (II, III) and Vertical * Sagittal to predict MYO1C expression. A second model was also created consisting of the group (Vertical * Sagittal) to predict MYO1C expression.

4.4.2 Kendall's Tau-b Correlation Analysis

The second analysis consisted of creating correlations using Kendall's Tau-b. A tau test is a non-parametric hypothesis test which uses the coefficient to test for statistical dependence. Given the small sample sizes, the Tau test is most appropriate. The analysis examined the relationship between MYO1C expression and both myosin heavy chain

gene expression (I, IIA, IIX, IIA/IIX, Neo, Atrial, Neo-Atrial) and fiber type percent occupancy (Type I, Hybrid I/II, Type II, and Type Neo-Atrial).

4.4.3 Power Analysis

Finally, a power analysis was completed to determine the minimum effect size that is likely to be detected in the study using the given small sample size, as well as the sample size needed to detect significant differences. The sample size calculation indicates the following:

Using an eta-squared of 0.123 the standardized effect size = 0.374

Alpha = 0.05

Power = 0.80

Number of groups = 6

CHAPTER 5

RESULTS

5.1 Descriptive Statistics

42 masseter samples have undergone RNA extraction and real time PCR assays to quantify MYO1C. Fiber type analysis was also performed for each of the samples to determine if a correlation exists between gene expression and fiber occupancy. Table 1 lists the descriptive data for age, sex and malocclusion groups of the entire sample. All growth factor expression data can be found in Appendix A, Myosin Heavy Chain Gene Expression Data in Appendix B and Masseter Muscle Fiber Percent Occupancy Data in Appendix C. No statistically significant differences were seen with the ANOVA analysis for age and gender between malocclusion groups (both individual and combined sagittal-vertical), as well as when age and gender were removed as variables. The P values are as follows: 0.332 for vertical malocclusion, 0.326 for sagittal malocclusion and 0.442 for combined vertical * sagittal malocclusion. Significant correlations were found, however, between MYO1C and both MyHC gene expression and fiber type percent occupancy. The power of the study was only 36% due to the small sample size. In order to find significant differences, a sample size of 102, or 17 per group is necessary.

5.2 Expression of MYO1C by Vertical and Sagittal Dimensions

The following figures show the mean and standard error for expression of MYO1C by both sagittal and vertical dimensions. Analysis of MYO1C expression by

vertical dimension revealed increased expression of the protein in open and normal bite individuals in comparison to deep bite individuals (Figure 4). Figure 5 shows that MYO1C had slightly greater expression in Class III masseter samples.

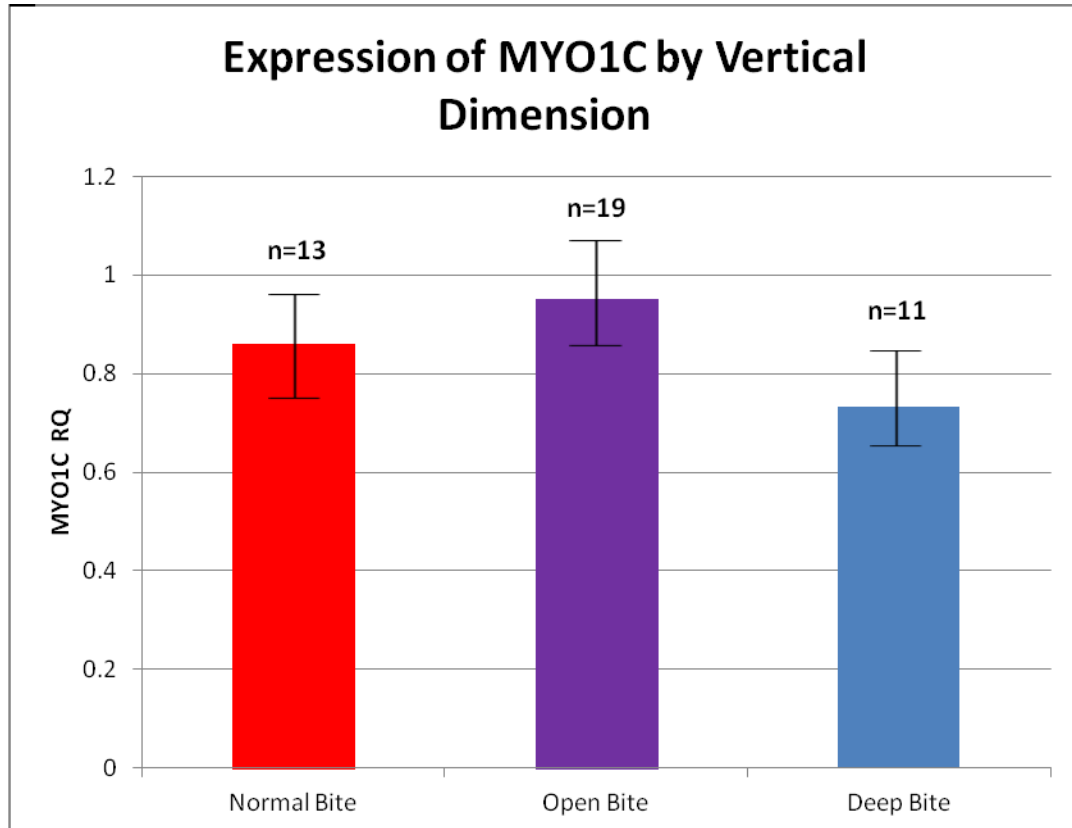


Figure 4. Expression of MYO1C Compared by Vertical Dimension

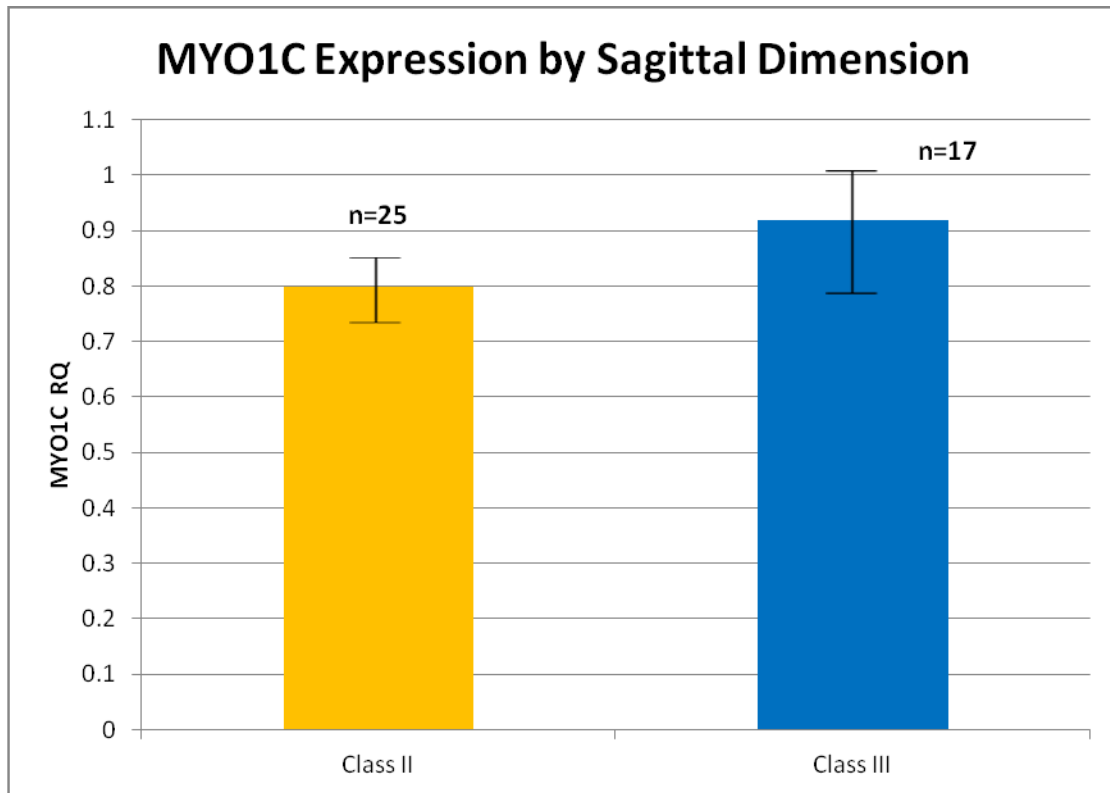


Figure 5. Expression of MYO1C Compared by Sagittal Dimension

MYO1C expression was also evaluated by combined vertical and sagittal malocclusion groups. Relative quantities (RQ) of RNA are shown in Table 3 and plotted in Figure 6. The highest expression is seen in Class III open bite patients with the lowest in Class II normal bites.

Table 3. Means and Standard Deviations of MYO1C Expression Compared by Occlusal Group

Vertical		Sagittal	
		2	3
Deep Bite	M	0.78	0.68
	SD	0.27	0.41
Normal Bite	M	0.64	0.99
	SD	0.33	0.38
Open Bite	M	0.89	1.03
	SD	0.38	0.62

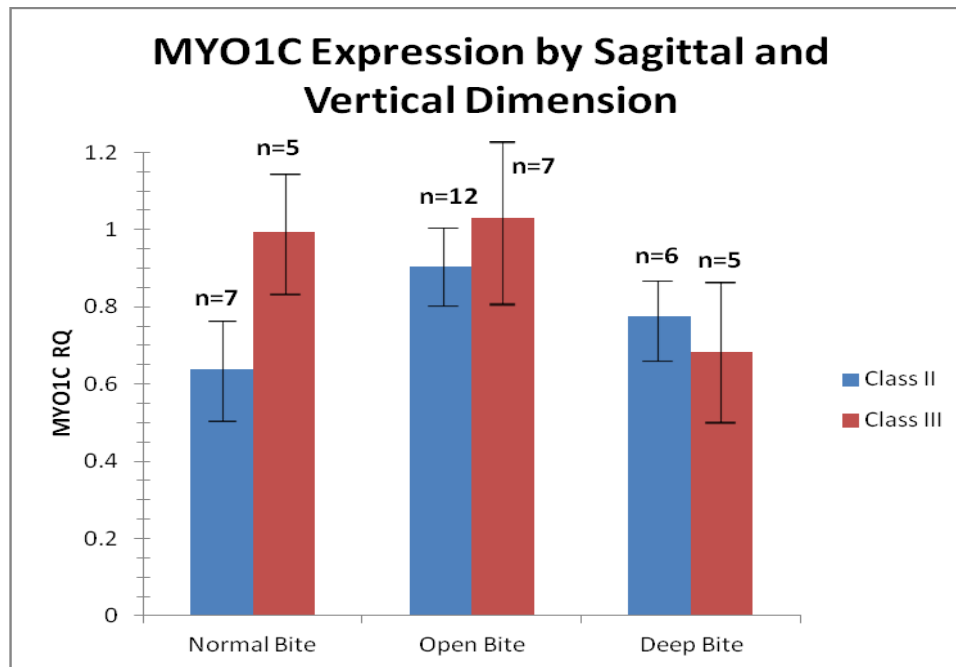


Figure 6. Comparison of Expression of MYO1C by Combined Vertical and Sagittal Malocclusion Groups

5.3 Correlation Analysis of MYO1C Expression to Myosin Heavy Chain Gene Expression and Mean Percent Occupancy Data for Fiber Types

The second aim of this thesis project was to evaluate whether or not a correlation exists between MYO1C expression and either myosin heavy chain gene expression and fiber-type occupancies (Appendix E). Using Kendall's Tau-b analysis, significance ($p < 0.05$) was found for 7 correlations between MYO1C and MyHC expression and for 5 correlations with fiber occupancies shown in Figures 7-18 below.

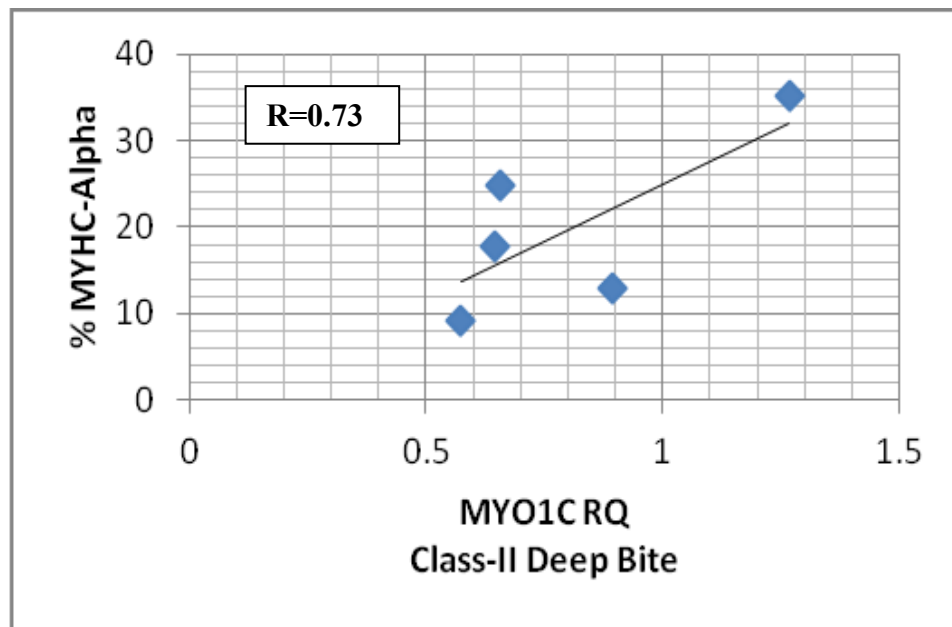


Figure 7. Correlation of MYO1C to Atrial Myosin Gene Expression in Masseter Muscle from Class II Deep Bite Subjects

Comparison of MYO1C to atrial myosin heavy chain (MHC) gene expression in Class II deep bites showed a high correlation of $R=0.73$.

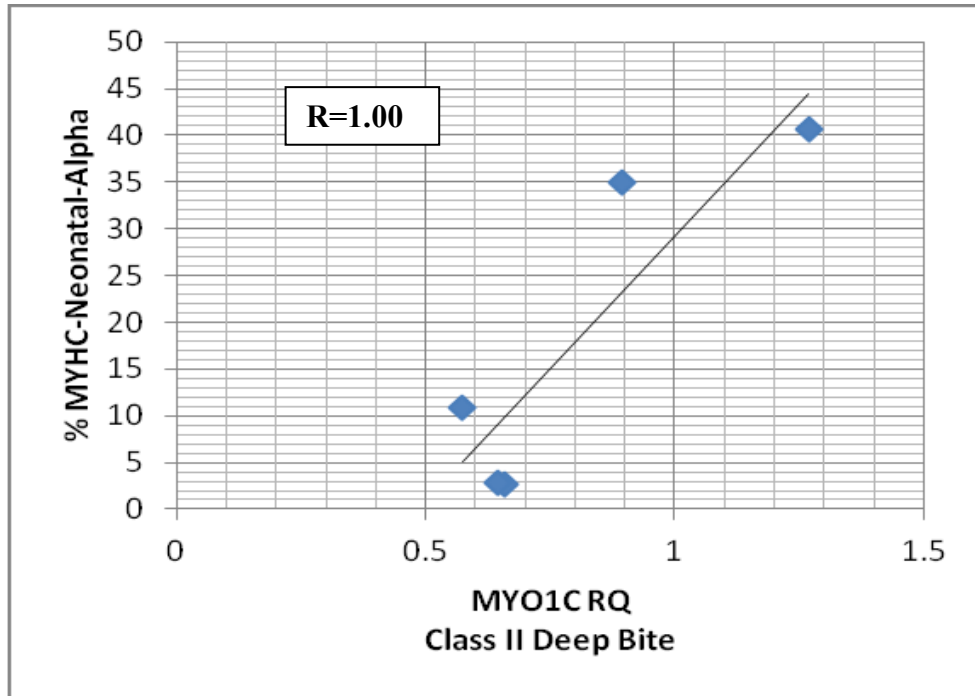


Figure 8. Correlation of MYO1C to Neonatal-Atrial Myosin Gene Expression in Masseter Muscle from Class II Deep Bite Subjects

Comparison of MYO1C to neonatal-atrial MHC expression in Class II deep bites showed a high correlation with $R=1.00$.

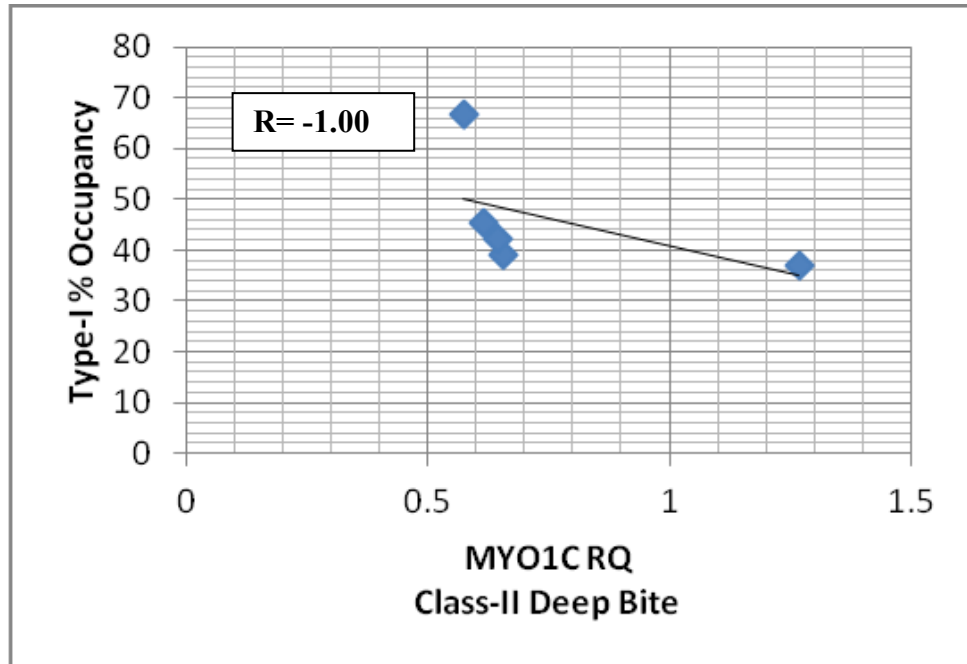


Figure 9. Correlation of MYO1C to Type I Fiber Percent Occupancy in Class II Deep Bites

Comparison of MYO1C to type I fiber percent occupancy in Class II deep bites showed a high negative correlation with $R = -1.00$.

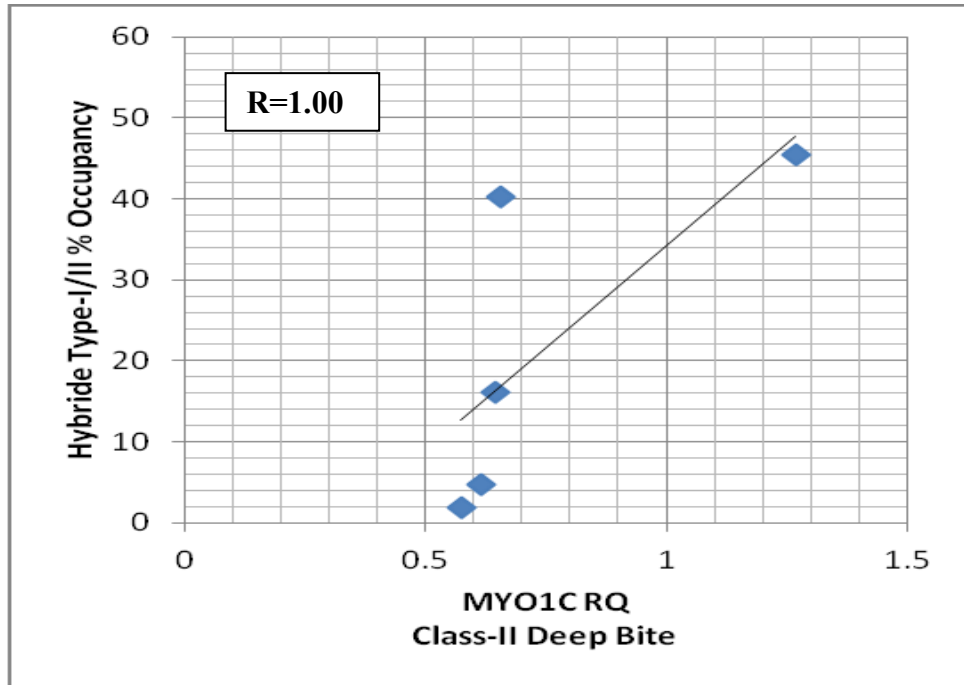


Figure 10. Correlation of MYO1C to Hybrid Type I/II Fiber Percent Occupancy in Class II Deep Bites

Comparison of MYO1C to hybrid Type I/II fiber percent occupancy in Class II deep bites showed a high correlation with $R=1.00$.

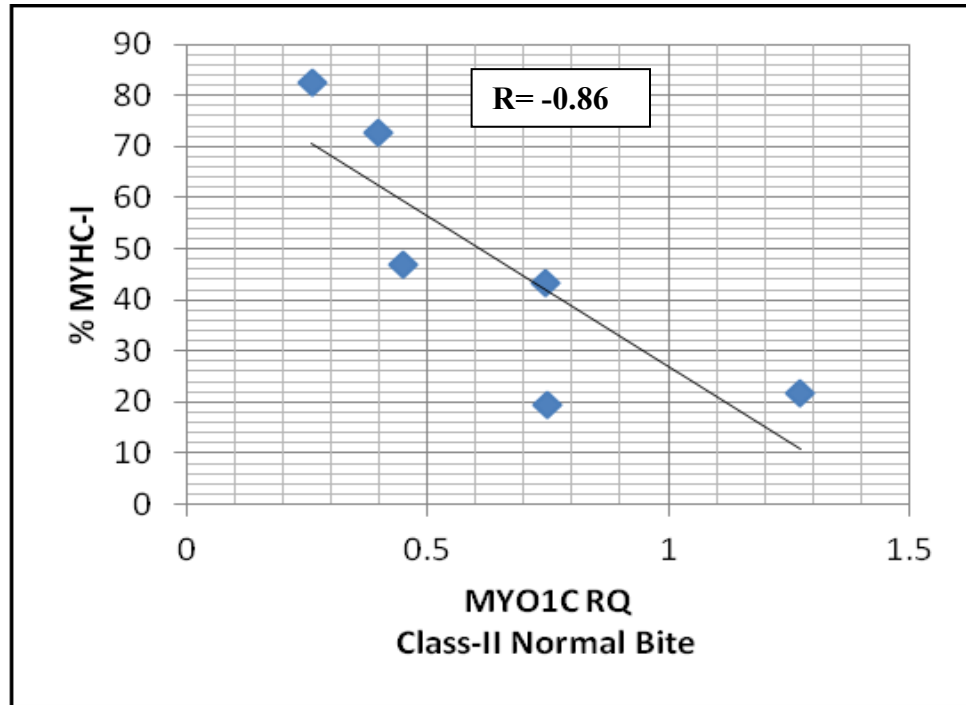


Figure 11. Correlation of MYO1C to Type I Myosin Gene Expression in Class II Normal Bites

Comparison of MYO1C to type I MHC gene expression in Class II normal bites showed a high negative correlation with $R = -0.86$

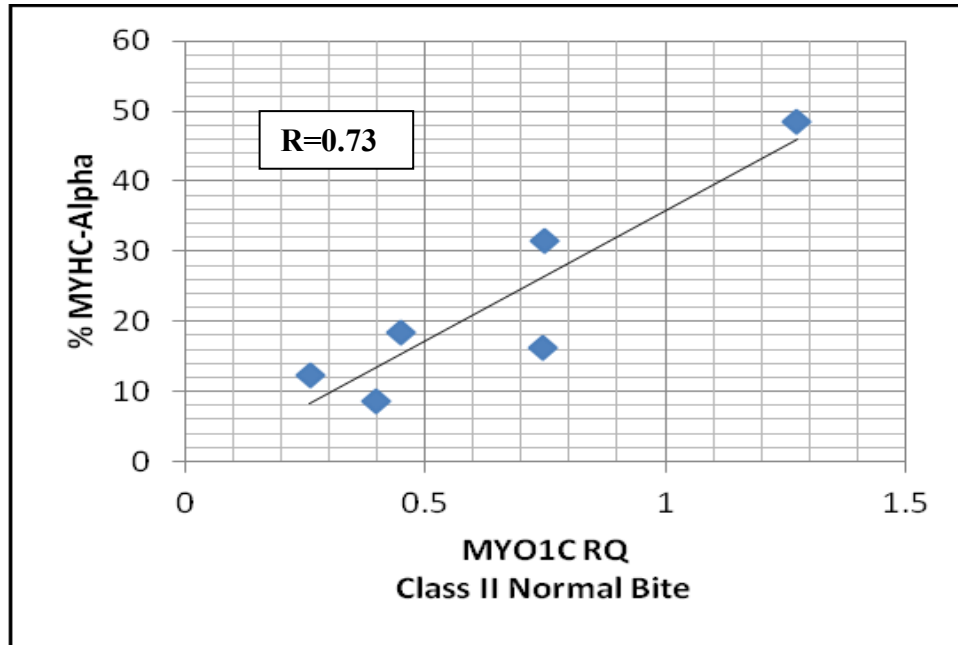


Figure 12. Correlation of MYO1C to Atrial Myosin Gene Expression in Class II Normal Bites

Comparison of MYO1C to atrial MHC gene expression in Class II normal bites showed a high correlation with $R= 0.73$.

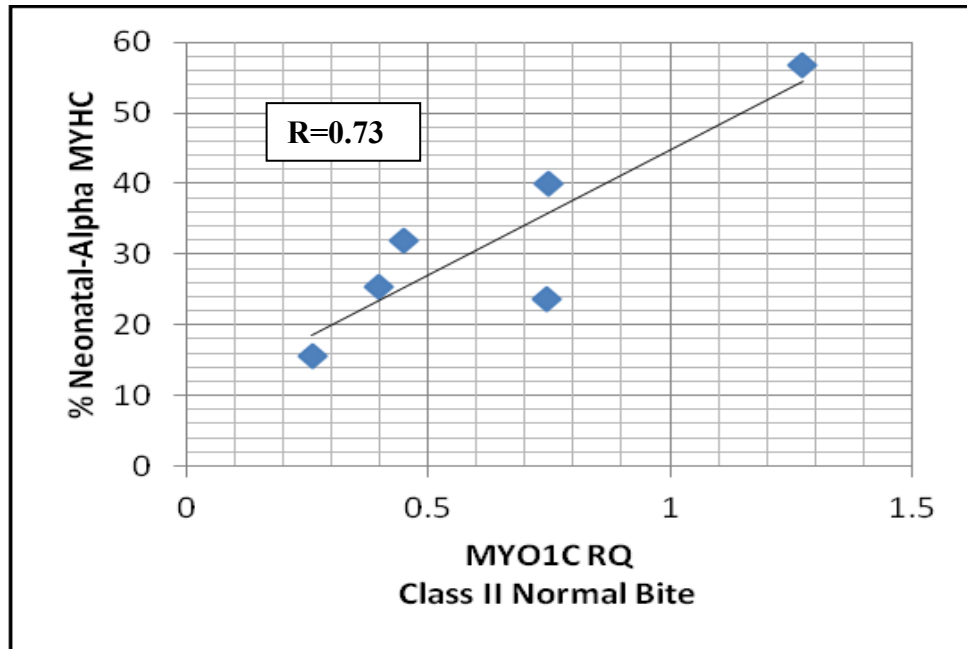


Figure 13. Correlation of MYO1C to Neonatal-Atrial Myosin Gene Expression in Class II Normal Bites

Comparison of MYO1C to neonatal-atrial MHC gene expression in Class II normal bites showed a high correlation with $R= 0.73$.

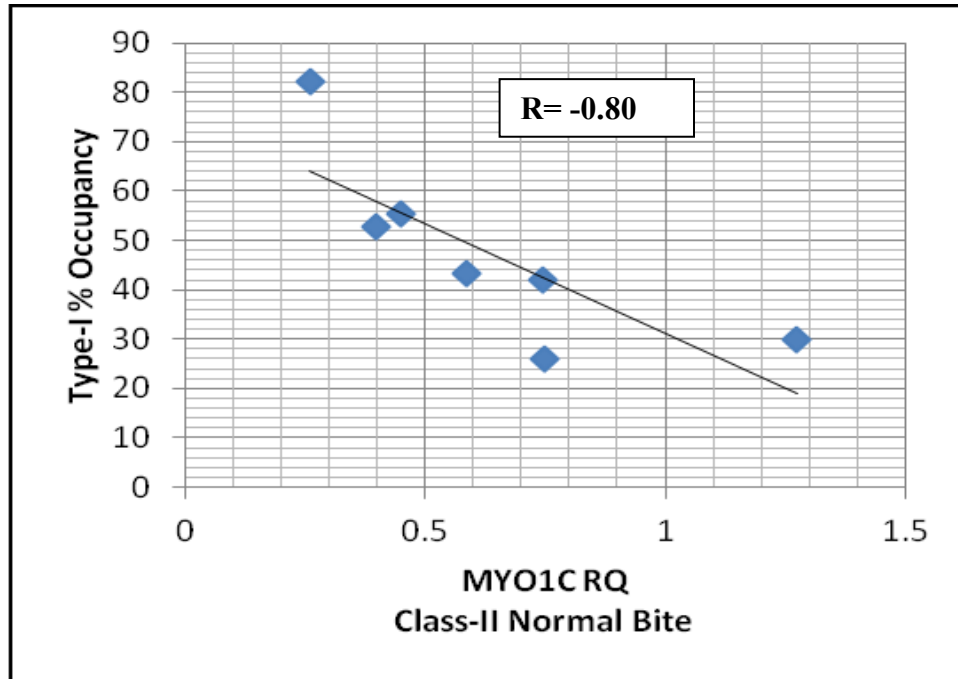


Figure 14. Correlation of MYO1C to Type I Fiber Percent Occupancy in Class II Normal Bites

Comparison of MYO1C to type I fiber percent occupancy in Class II normal bites showed a high negative correlation with $R = -0.80$.

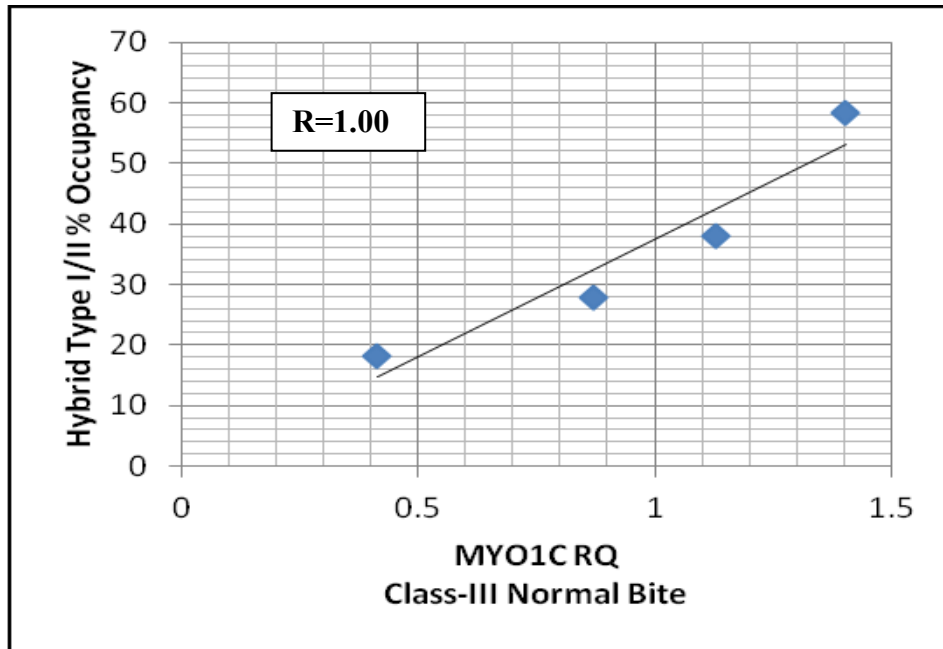


Figure 15. Correlation of MYO1C to Hybrid Type I/II Fiber Percent Occupancy in Class III Normal Bites

Comparison of MYO1C to hybrid type I/II fiber percent occupancy in Class III normal bites showed a high correlation with $R= 1.00$.

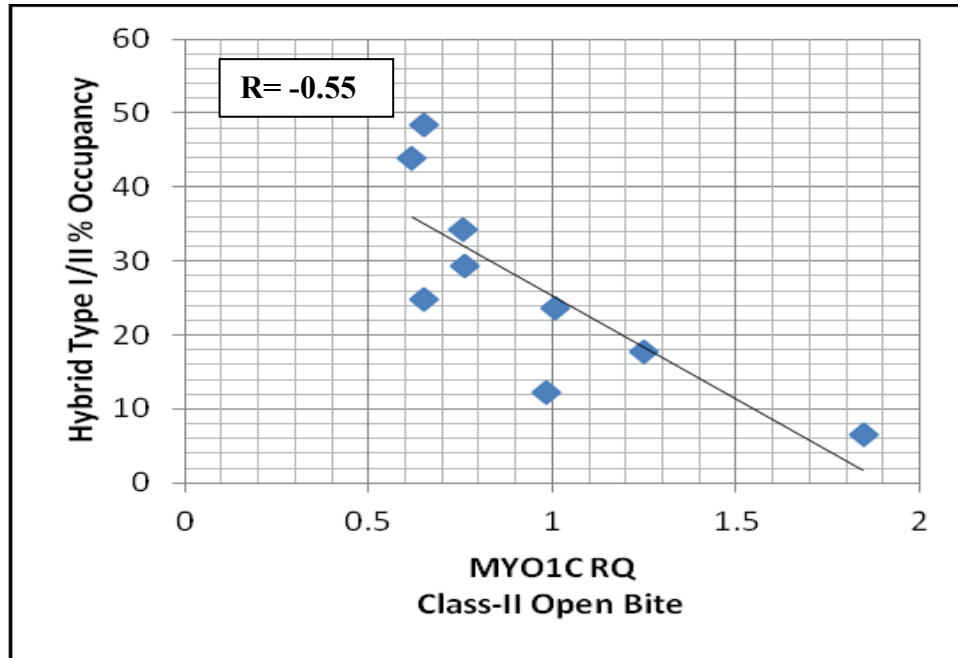


Figure 16. Correlation of MYO1C to Hybrid Type I/II Fiber Percent Occupancy in Class II Open Bites

Comparison of MYO1C to hybrid type I/II fiberpercent occupancy in Class II open bites showed a high negative correlation with $R= -0.55$.

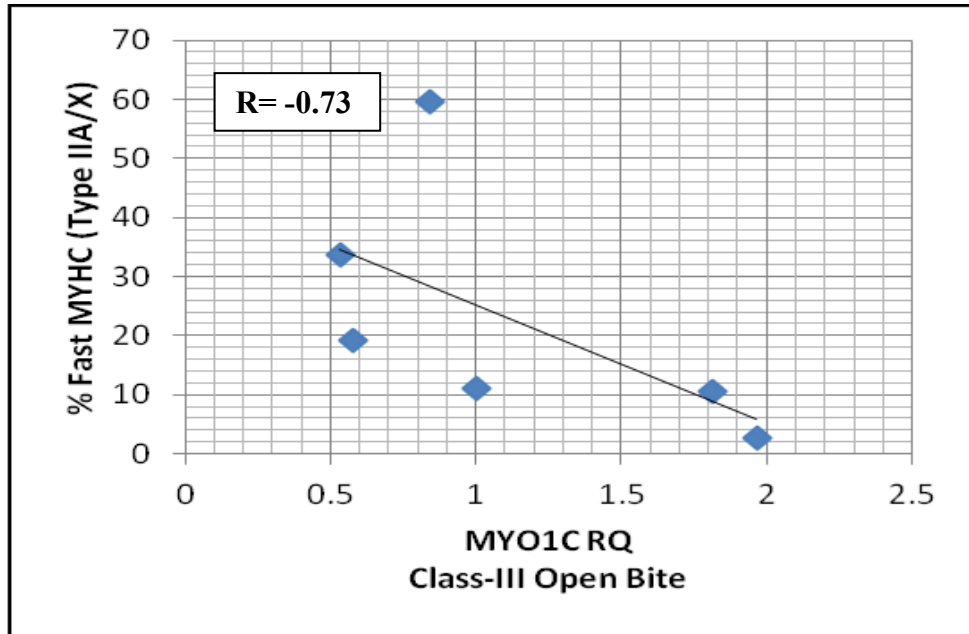


Figure 17. Correlation of MYO1C to Type IIA/X Myosin Gene Expression in Class III Open Bites

Comparison of MYO1C to type IIA/X MHC gene expression in Class III open bites showed a high negative correlation with $R = -0.73$.

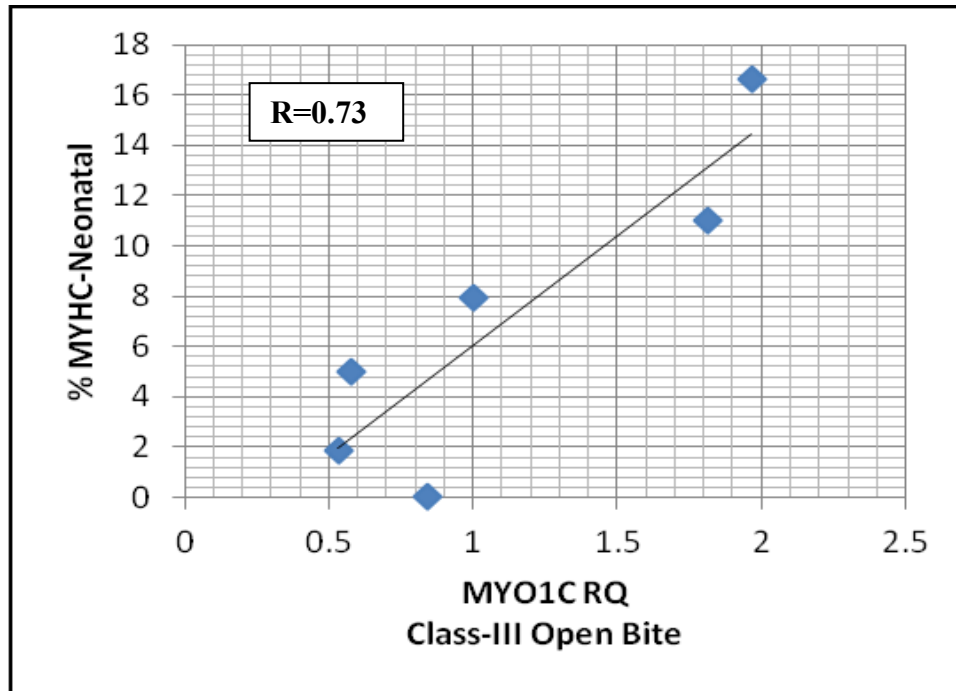


Figure 18. Correlation of MYO1C to Neonatal Myosin Gene Expression in Class III Open Bites

Comparison of MYO1C to neonatal MHC gene expression in Class III open bites showed a high correlation with $R=0.73$.

5.4 Comparison of MYO1C to MSTN and IGF-1 Expression Among Malocclusion Groups

Expression of MYO1C to MSTN and IGF-1 was also compared to evaluate whether similar trends in expression existed among different malocclusion groups. Overall, the expression of MYO1C was markedly less than that of MSTN and IGF-1, indicating less prevalence in masseter muscles. RQ values were plotted in Figure 19, but

the graph alone does not enable a good analysis of correlation between MYO1C and the two growth factors due to the vast differences in expression quantity.

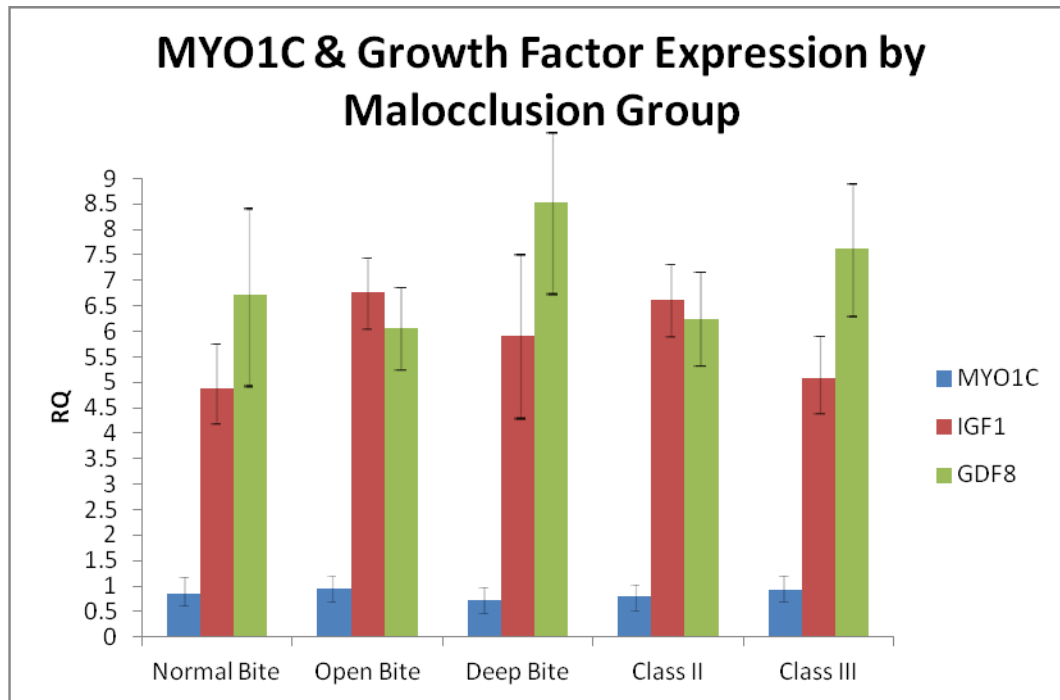


Figure 19. MYO1C & Growth Factor Expression by Malocclusion Group

In order to better evaluate any relationship between MYO1C and growth factor expression, correlation coefficients were calculated (Appendix F). After analysis between MYO1C and both IGF-1 and MSTN using Pearson's coefficient of correlation, three significant correlations were noted ($p < 0.05$) and the graphs are shown below with descriptions.

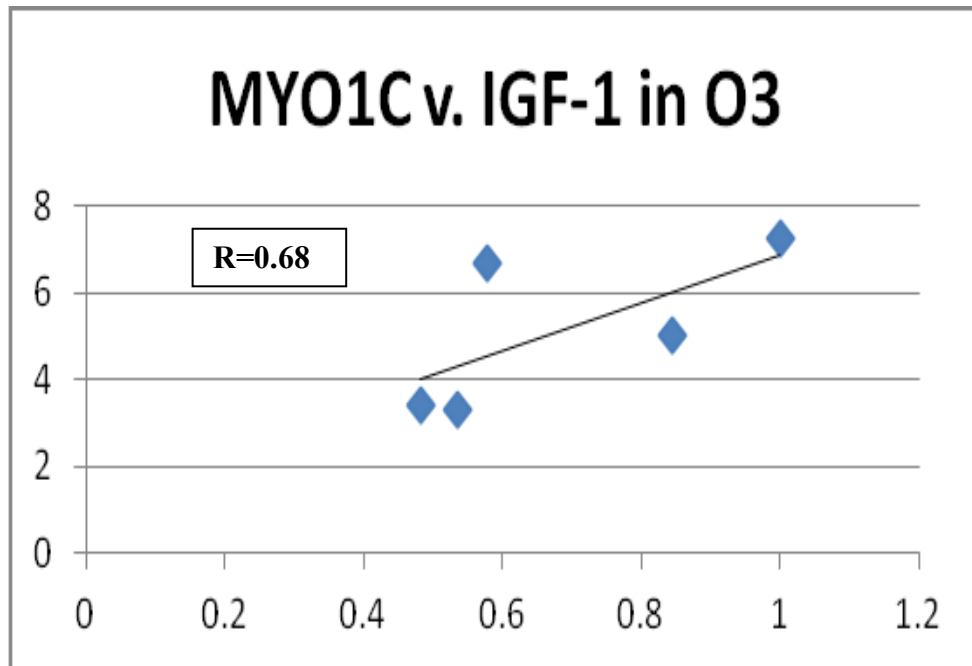


Figure 20. Correlation of MYO1C to IGF-1 in Class III Open Bites

Comparison of MYO1C to IGF-1 expression in Class III open bites exhibited a high correlation with $R=0.68$.

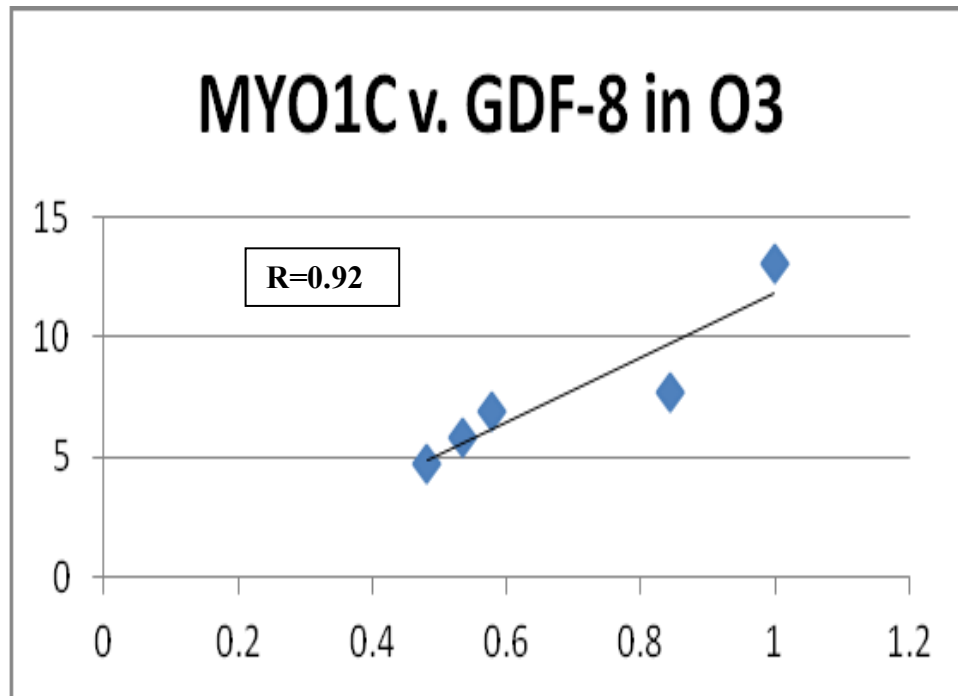


Figure 21. Correlation of MYO1C to GDF-8 in Class III Open Bites

Comparison of MYO1C to GDF-8 also showed a high correlation in Class III open bites with an R value of 0.92.

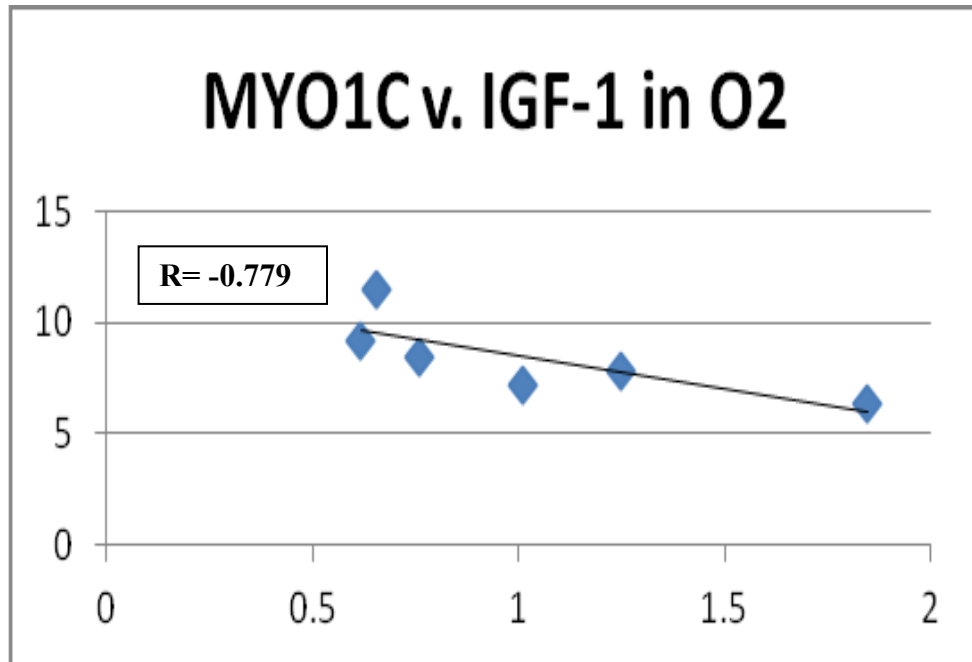


Figure 22. Correlation of MYO1C to IGF-1 in Class II Open Bites

Comparison of MYO1C to IGF-1 in Class II open bites showed a high inverse correlation with an R value of -0.779.

5.5 Comparison of MYO1C and MYO1H Expression Among Malocclusion Groups

MYO1H expression was quantified in only 11 samples due to inadequate amounts of RNA available for the rest of the samples. These 11 samples were compared by malocclusion group for expression of MYO1H to MYO1C. While the sample size was not large enough to generate statistical significance, some interesting trends were noted. MYO1H exhibited greater expression than MYO1C in all malocclusion groups except

deep bites, particularly Class II deep bite samples. Expression of the two MYO1 types in general was significantly different in Class III, normal and open bite samples, with MYO1H being greater (Figure 23).

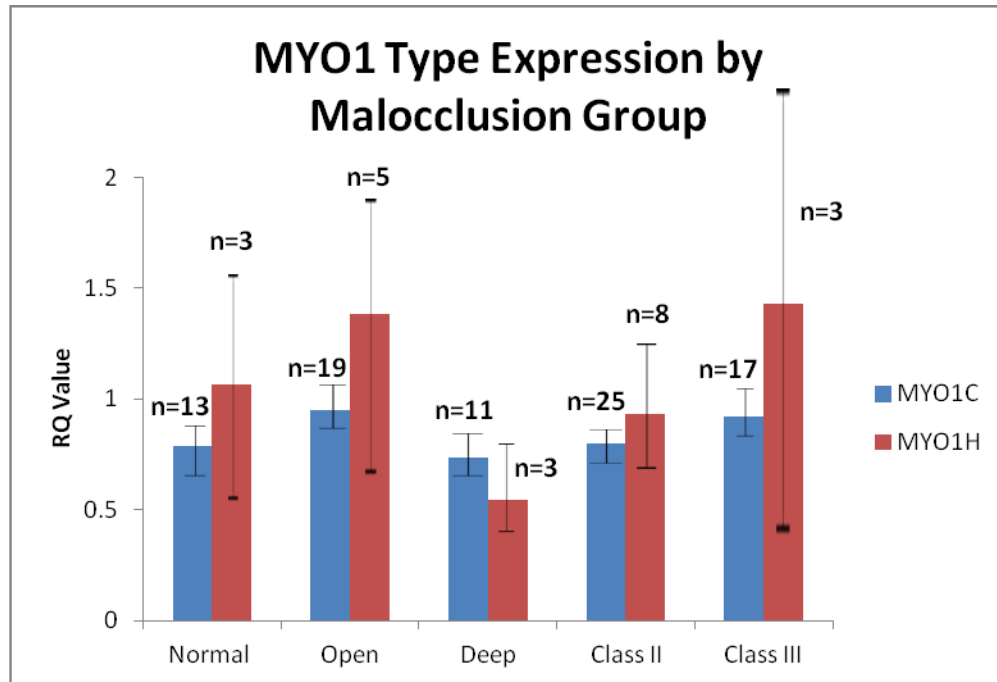


Figure 23. Comparison of MYO1 Type Expression by Malocclusion Group

When comparing the combined malocclusion groups, MYO1H had significantly greater expression in Class III open bite samples, while MYO1C had greater expression in Class II deep bite samples. The two genes seemed to exhibit similar expression patterns only in Class II open bite samples (Figure 24).

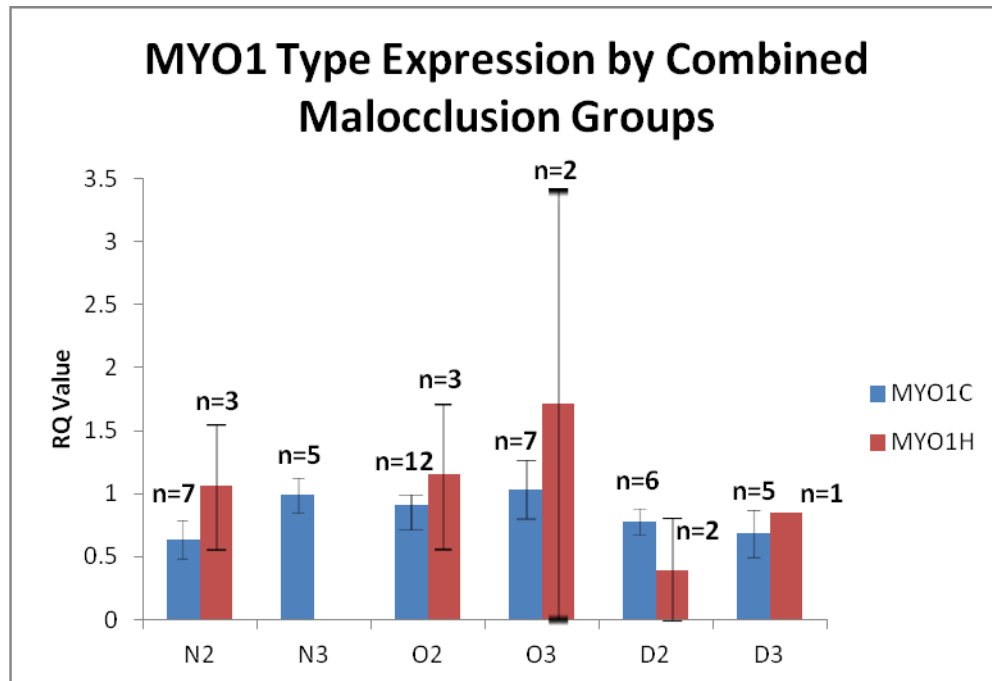


Figure 24. Comparison of MYO1 Type Expression by Combined Malocclusion Groups

5.5.1 Comparison of MYO1C and MYO1H Expression by Myosin Heavy Chain Gene Expression and Percent Occupancy

In order to determine whether the expression patterns of MYO1C and MYO1H were different among fiber types, correlations were calculated between each of the respective proteins and both myosin heavy chain gene expression and fiber percent occupancy for the entire sample (Appendix G). These correlation values were calculated using Pearson's coefficient of correlation and were compared to evaluate differences by fiber type. The correlation coefficients, however, were very low indicating non-significant results.

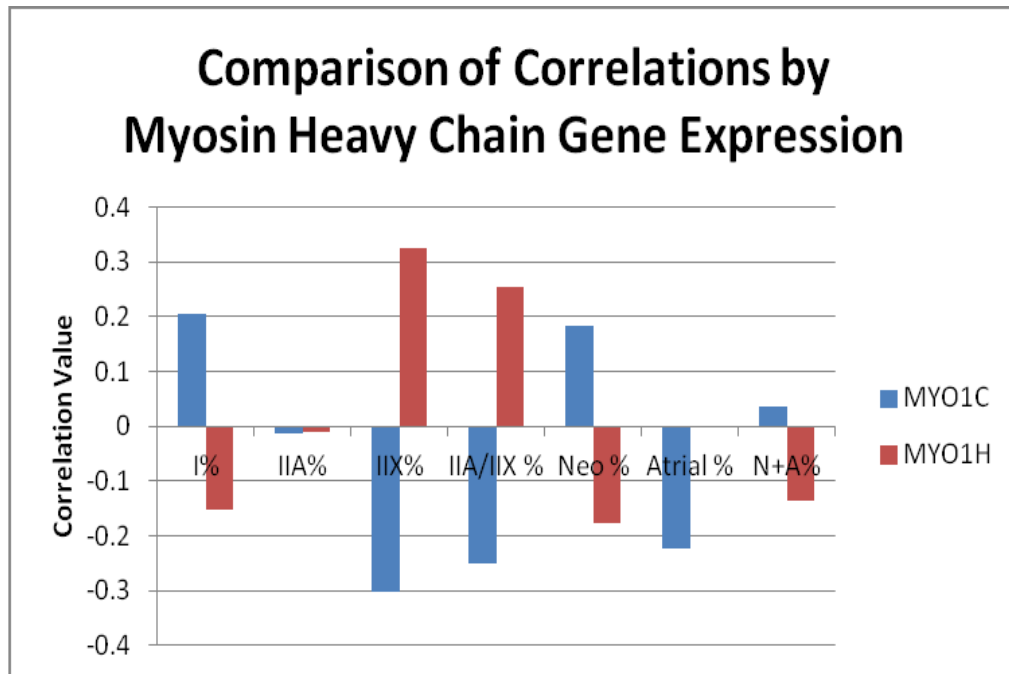


Figure 25. Comparisons of Correlations by Myosin Heavy Chain Gene Expression

Figure 25 compares the correlation of each of the two genes to myosin heavy chain gene expression. MYO1C exhibits a strong positive correlation to Type I and Neonatal MHC gene expression, while MYO1C exhibits a strong positive correlation to Type IIX and combined Type IIA/X MHC gene expression. Interestingly, MYO1C and MYO1H showed almost complete opposite correlations for all fiber types except Type IIA.

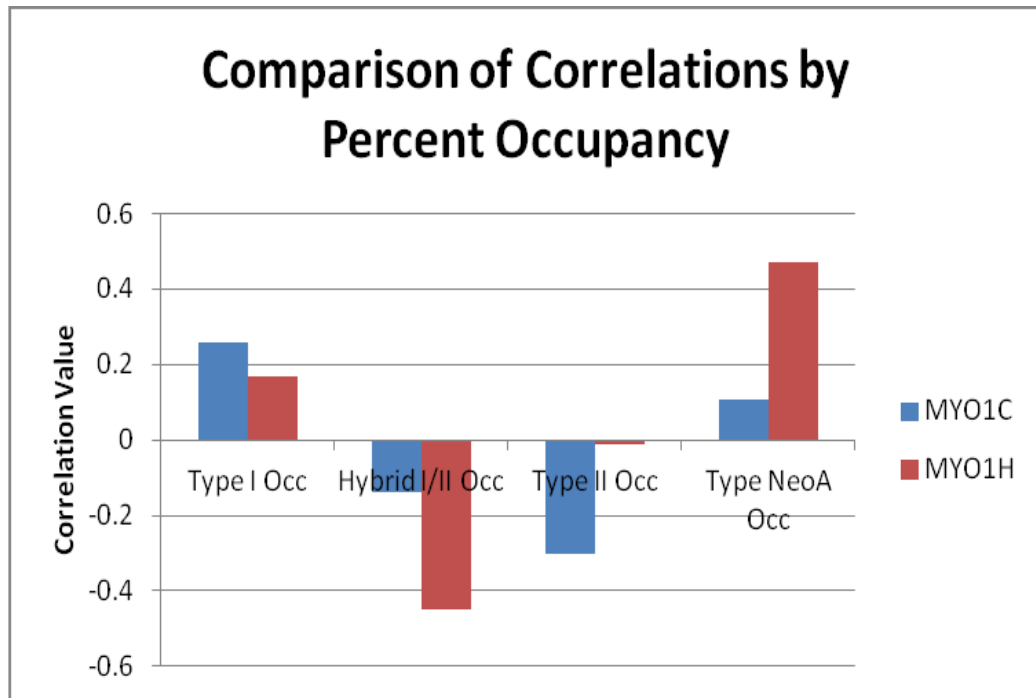


Figure 26. Comparison of Correlations by Fiber Type Percent Occupancy

Figure 26 compares the correlation values of both MYO1 types to fiber percent occupancy. MYO1C had the greatest correlation to Type I fiber percent occupancy, while MYO1H was most greatly correlated with Type Neo-Atrial fiber percent occupancy. A negative correlation was indicated for both the Hybrid I/II and Type II percent occupancy. The R values, however, were too low to indicate significance.

CHAPTER 6

DISCUSSION

Due to the prevalence of malocclusion in today's population, it is imperative to identify underlying causes in order to both improve diagnosis and possibly prevent handicapping malocclusions requiring surgical intervention later in life. The etiology of malocclusion is complex, comprised of behavioral, environmental, and genetic components, yet least is known about the genetic piece to the puzzle. Numerous studies have confirmed the correlation between skeletal form and muscle function, with muscle fiber composition being a significant factor in ultimately affecting skeletal development. Rowlerson et al. (2005) showed that fiber type composition varied greatly between open and deep bite subjects; the size and occupancy of type I fibers differed little in open bite subjects compared to normal, but the occupancy of type II fibers drops to 8%. Conversely, deep bite subjects showed decreased occupancy of type I fibers and a substantial increase in type II fiber occupancy. The proportions of these fibers greatly affect the phenotype of the muscles as well as their oxidative capacity. Type I fibers have slow shortening velocities and are fatigue resistant, as they produce ATP via oxidative metabolism. In contrast, type II fibers have rapid shortening velocities but fatigue relatively easily due to the relatively less production of ATP via glycolysis. Needing more glucose to produce the same amount of ATP, type II fibers are more sensitive to the uptake of glucose subsequent to insulin release than type I fibers.

In this study, we analyzed the expression of MYO1C, a protein that regulates glucose uptake in skeletal muscle cells by acting as a motor for movement of GLUT4-

stored vesicles to plasma membranes (Toyoda et al., 2010). MYO1C expression is upregulated in association with increased muscle contraction, insulin-stimulated glucose uptake and increased oxidative capacity. As a major player in the control of glucose uptake into muscle, MYO1C may mediate the metabolic effects of growth factors on fiber size and phenotype. In doing so, skeletal growth and form will be affected.

This study aimed to examine what effect MYO1C has on skeletal growth pattern, whether a correlation does exist between MYO1C expression and fiber type composition, and how the expression of MYO1C compares to that of growth factors (MSTN and IGF-1) and MYO1H. While several of the results showed promising data trends, one of the limitations of this study was the inability to obtain larger sample numbers, which would have helped increase the statistical significance of many of the results. A power analysis was completed following completion of data collection, which found the study's power to be 36%. The total sample needed to find significant differences is 102 or 17 per group, when divided into 6 occlusal groups (combined vertical-sagittal). Given the power of this preliminary study, future studies can be executed with an increased sample size to guarantee significance.

6.1 Expression of MYO1C by Occlusal Group

The results obtained from our RT-PCR assays indicate that MYO1C is more prevalent in samples from open and normal bite than deep bite samples. A major function of MYO1C is the facilitation of GLUT4 vesicle transport for uptake of glucose into cells.

Accordingly, muscle fibers with high oxidative capacity that are heavily dependent on glucose are expected to express relatively more MYO1C. Established oxidative metabolic profiles of muscle fibers are known to be type I > type IIA > type IIX. In contrast, glycolytic profiles are type IIX > type IIA > type I. Overall, type I fibers are considered to have high oxidative and low glycolytic capacity whereas type IIA fibers have both high oxidative and glycolytic properties. Rowlerson et al (2005) showed that masseter muscles in open bite patients have a greater percent occupancy of type I than type II fibers. Since type I fibers have a superior oxidative capacity, it is not surprising to find that MYO1C expression would be higher in masseter of open bite subjects. The lower expression of MYO1C in deep bite samples may also indicate that there is a reduced amount of glucose uptake by the type II fibers which could consequently be correlated to the fatigability of these fibers.

When comparing the sagittal malocclusion, expression of MYO1C was found to be higher in Class III samples versus Class II. Tassopoulou et al. (2011; 2012) showed an association between prognathism, a Class III malocclusion, and a single nucleotide polymorphism (SNP) in the MYO1H gene. Because MYO1H is a vertebrate-specific paralog of MYO1C, one might speculate whether MYO1H expression could supplement the metabolic or transcriptionally-related functions of MYO1C between different occlusal types. Patterns of expression between these two myosins indicate that MYO1C is the much more abundant species, so that whatever small functional contribution of a polymorphic MYO1H is present might be potentially magnified if MYO1C were down-regulated in malocclusion. Alternatively, expression of MYO1H, whether in wild-type or

polymorphic form, during early development might elicit a more substantial effect on craniofacial phenotype resulting in eventual postnatal malocclusion.

Two-way ANOVA models were created to analyze the data for significance by both individual occlusal groups (deep, normal, open, Class II, Class III) and combined occlusal groups (vertical * sagittal). No significant differences were found, as the P values were greater than 0.05 as listed: 0.332 for vertical malocclusion, 0.326 for sagittal malocclusion and 0.442 for combined vertical * sagittal malocclusion. As stated above, a greater sample size of approximately 102 would permit an accurate test for significant differences.

6.2 Correlation of MYO1C to Fiber Type

When correlating MYO1C expression to myosin heavy chain gene expression and fiber type percent occupancy, several significant correlations were found. Because differences exist in the sensitivity of muscle fibers to glucose uptake, one would expect to see variable expression of genes related to glucose transport between these fiber types. MYO1C functions in glucose transport and has been shown to increase both contraction- and insulin-stimulated glucose uptake (Toyoda et. al, 2011). The level of this protein may well contribute to the metabolic phenotype of the muscle, with its abundance being higher in more oxidative skeletal muscle and the heart. Muscle of both Class II normal and deep bite subjects showed a high correlation of MYO1C to both atrial and combined neonatal-atrial MHC gene expression. While not found in limb skeletal muscle, atrial

fibers are found in human masseter muscles but the frequency and distribution is uncertain. Because MHC- α , which was assayed here, is expressed in fiber types of both masseter muscle and the heart atrium, it follows that atrial muscle fibers have higher oxidative capacity and thus increased expression of MYO1C.

Class III open bites exhibited a high positive correlation between neonatal myosin gene expression and MYO1C while having a negative correlation between type II MHC gene expression and MYO1C. Open bite masseter samples have a low percent occupancy of type II fibers (Rowlerson et al., 2005), which require less glucose in comparison to type I fibers. In a study measuring glucose uptake in single muscle fibers, MacKrell et al (2012) found that type IIA fibers have two-fold greater glucose uptake versus IIB or IIX due to their superior oxidative capacity. Type I fibers were not included in this assay, but would be expected to have greater glucose uptake than all type II fibers. These findings support our data in Class III open bites, where MYO1C would increase glucose uptake necessary for type I fiber metabolism. On the other hand, Class II normal and deep bite samples showed a negative correlation between type I MHC gene expression and type I fiber percent occupancy and MYO1C. These results can be explained by the fact that deep bite samples have a low percent occupancy of type I fibers, so the abundance of MYO1C needing to regulate glucose uptake would be expected to decrease.

Of all the fiber types, the percent occupancy of hybrid I/II fibers was most varied and least predictable when correlated to MYO1C expression. While Class III normal and Class II deep bites showed a positive correlation between hybrid I/II fiber percent occupancy and MYO1C, Class II open bites exhibited a negative correlation. Because

hybrid fibers are transitional fibers between type I and II, this may account for the random nature of these results.

In a study of GLUT4 along with four of its regulatory proteins, relationships with fiber types in several muscles of rats were reported (Castorena et al., 2011). Significant positive correlations were found between the %MHC I and GLUT4 protein ($R = 0.460$), the tethering protein containing an UBX-domain protein (TUG; $R = 0.538$) and the RuvB-like protein two (RUVBL2; $R = 0.511$). For the % MYH-IIA, positive correlations were found with GLUT4 ($R = 0.293$) and RVUBL2 ($R = 0.204$). Notably, correlations with very fast contracting rodent MYH-IIB fibers were negative with GLUT4 ($R = -0.642$), TUG ($R = -0.626$) and RVULB2 ($R = -0.692$). These results, like those seen in our study of MYO1C, suggest that the expression of GLUT4 and genes for proteins involved with GLUT4 function is relatively enhanced in predominately oxidative fibers, but diminished in the fast fiber classes IIX in human and IIB in rat.

6.3 Correlation of MYO1C to Growth Factors

Previous studies have demonstrated the effects of growth factors on skeletal growth dimension. MSTN is a negative regulator of muscle growth which functions to inhibit hypertrophy when expressed in normal quantities. When studying MSTN-knockout mice, Vecchione et al. (2007; 2010) found significantly greater body and muscle weight as well as brachycephalic crania with smaller cranial dimensions compared to wild-type controls. IGF-1, on the other hand, is a positive growth regulator

of the growth hormone (GH)/IGF1 axis and mediates the muscle growth-promoting actions of GH (Philippou et al., 2007). Because of MSTN and IGF's known roles in affecting muscle metabolism and consequently skeletal development, one of the aims of this study was to evaluate if a correlation exists between expression of either of the growth factors and MYO1C.

When looking for differences between MYO1C and both IGF-1 and MSTN, we found that expression levels of both growth factors were much higher than MYO1C in all muscle samples. This result was expected due to the prevalence of these major growth factors in skeletal muscle. Correlation analyses showed a high positive correlation between MYO1C and both IGF-1 and MSTN in Class III open bites. Class II open bites, however, exhibited a negative correlation between MYO1C and IGF-1. As such, it seems as if the difference in expression is not related to vertical dimension since both were open bite samples, but rather sagittal dimension. Class III samples had coordinated expression of MYO1C and growth factors, indicating that growth factors may act in harmony with MYO1C in Class III masseter muscle metabolism. The opposite can be said of Class II samples, where expression of IGF-1 and MYO1C are not coordinated. Having also studied the expression of both IGF-1 and MSTN in masseter muscle samples from varying occlusal groups, Sciote et al (2012) found that the ratio of IGF-I:GDF-8 expression is associated with type I and II mean fiber areas, which differs by occlusal group. Type II mean fiber area is significantly higher in deep bite samples versus open bite, so it is clear that levels of growth factor expression influence fiber properties and thus differ by occlusal group.

6.4 Comparison of Expression between MYO1C and MYO1H

Although MYO1H is not itself a growth factor, the polymorphism of this gene reported by Tassopoulou et al. (2011; 2012) is linked to Class III malocclusion and could therefore have a significant effect on growth and function of muscular and skeletal components of the face. As discussed before, there may be an important link between functions of class I myosins and musculoskeletal development for sagittal jaw deformation. Because MYO1C is a paralog to MYO1H, we sought to determine its correlation to MYO1H by occlusal group in order to evaluate if their expression patterns were similar.

Expression levels were not significantly different, with the exception of greater relative quantities of MYO1H in Class III open and normal bites and MYO1C in Class II deep bites. The prevalence of MYO1H in Class III open bites corresponds to the studies by Tassopoulou et al., which linked MYO1H to Class III malocclusions. Furthermore, of all the occlusal groups, MYO1C had the highest expression in Class III open bites so it is evident that class I myosins are heavily expressed in this particular group. Having the lowest expression of MYO1H, Class II deep bite masseters appear to be less regulated by class I myosins.

In addition to comparing the expression levels of MYO1C and MYO1H, we also calculated the correlation values between the class I myosins and both myosin heavy chain gene expression and percent fiber occupancy. While not statistically significant due to inadequate sample size, several notable correlations were found. MYO1H exhibited a positive correlation to types IIA and IIA/X MHC gene expression and negative to types I,

neonatal and combined neonatal/atrial MHC gene expression. MYO1C, on the other hand, displayed a divergent profile with a positive correlation to types I and neonatal MHC gene expression and negative correlation to types IIA, IIA/X and atrial MHC gene expression. As such, it seems as if MYO1H is closely linked with type II MHC gene expression while MYO1C has a close association with types I and neonatal MHC gene expression. Fiber percent occupancy comparisons, on the other hand, displayed similar correlation patterns between MYO1C and MYO1H with a positive correlation for type I and neonatal/atrial fibers and a negative correlation for hybrid I/II and II fibers. In sum, these results indicate that although the two class I myosins showed distinct correlation patterns to MHC gene expression, there is an association relating both MYO1C and MYO1H to type I and neonatal/atrial fiber percent occupancy. Both class I myosins studied showed a predilection for Class III open bites, so it follows that a high correlation exists between MYO1C and MYO1H and type I fiber percent occupancy, which is increased in open bite samples.

6.5 Future Directions

Given the results of this study, it is prudent to look forward to determine how we, as professionals can use this information to advance the field of orthodontics. In order to increase the significance of the results, the first step to be taken is to replicate the study using a greater number of masseter samples from all six malocclusion groups. As stated above, the sample size for this study was limited to 42, which, while many data trends were discovered and several significant correlations noted, was inadequate to generate

significance for differences in MYO1C expression by occlusal group. Once more masseter samples are secured and assayed for MYO1C expression, concrete conclusions can be made regarding expression patterns by occlusal group.

As orthodontists, one of the most difficult tasks in treatment planning is correctly diagnosing young patients who still have significant growth potential remaining. While family history of skeletal malocclusion provides a useful guideline for estimating future growth, the field of orthodontics would greatly benefit if doctors could test for specific genes in patients to determine if a genetic predisposition to skeletal malocclusion exists. This study served as a preliminary investigation into expression of genes essential to masseter metabolism which may subsequently have an effect on skeletal growth. Given the promising results of this report, the hope for the future is to be able to perform genome studies on saliva samples from prospective patients. From these assays, the doctor will be able to evaluate the expression of genes involved in masseter growth and metabolism to translate orthodontic diagnosis and dictate a more appropriate treatment plan. With all the advances in medicine made possible by genomic research, the field of orthodontics holds endless potential for the future.

CHAPTER 7

CONCLUSIONS

-MYO1C expression is greater in open and normal versus deep bites and Class III versus Class II malocclusions. The highest expression is seen with Class III open bites and the lowest with Class II normal malocclusions.

- Class II deep and normal bites showed high correlation between MYO1C expression and atrial and neonatal/atrial MHC gene expression, which require increased MYO1C for oxidative metabolism. They exhibited a negative correlation to type I MHC gene expression and percent occupancy, as deep bites have fewer type I fibers.

-Class III open bites had high correlation between MYO1C and neonatal MHC gene expression and low correlation to type II MHC gene expression due to increased percentage of high oxidative type I fibers in open bites and diminished type II fibers.

-Correlations between MYO1C and hybrid I/II fiber percent occupancy was unpredictable by occlusal group due to transitional nature of fibers.

-MYO1C expression is correlated to growth factor expression in Class III but not in Class II malocclusions, indicating its potential interactive role in masseter metabolism in the Class III group.

-Class I myosins are highly expressed in Class III open bites.

-Class II deep bites exhibited the lowest expression of MYO1H, indicating the masseters are less regulated by class I myosins.

-MYO1H is closely linked with type II MHC gene expression, while MYO1C has a close association with types I and neonatal MHC gene expression.

-An association exists between class I myosins and both type I and neonatal/atrial fiber percent occupancy.

- A greater sample size of approximately 102 would permit an accurate test for significant differences in future studies.

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APPENDICES

APPENDIX A

Growth Factor Expression Data

<u>Sample</u>	<u>Sex</u>	<u>Age</u>	<u>Vert</u>	<u>Sagitt</u>	<u>MYO1C</u> <u>RQ</u>	<u>IGF1 RQ</u>	<u>GDF8</u> <u>RQ</u>	<u>MYO1H</u> <u>RQ</u>
1	F	25	N	2	0.450896293	3.3698769	4.1672816	1.76267087
2	M	17	N	3	0.413070679	2.8790464	4.966886	—
3	F	13	N	2	0.399792761	6.9832234	6.4226942	—
4	F	15	N	3	1.128490806	4.8912811	3.3111749	—
5	M	16	N	2	0.260094643	4.8803782	6.488358	1.37679385
6	F	15	N	2	0.744078636	2.9228557	4.8453128	0.04740776
7	M	20	N	3	1.402128696	—	—	—
8	M	16	N	2	0.584660828	—	—	—
9	M	29	N	2	0.747142375	8.2712021	16.844664	—
10	F	18	N	2	1.273348808	—	—	—
11	F	17	N	3	0.871286213	—	—	—
12	F	12	N	1	1.759899735	—	—	—
13	F	15	N	3	1.15382421	—	—	—
14	M	18	O	3	0.999677896	7.2780008	13.056429	0.00433261
15	F	16	O	3	0.577373743	6.6750426	6.8897848	—
16	F	18	O	2	1.009588838	7.239387	2.8055847	1.03824367
17	F	19	O	2	0.617408693	9.2330894	5.828546	—
18	F	20	O	2	1.249668956	7.7892556	2.5832822	0.22366955
19	F	15	O	3	0.48182857	3.428965	4.669999	—
20	F	17	O	2	0.835226178	—	—	—
21	F	32	O	3	0.534011722	3.3037183	5.8248711	3.42992359
22	F	18	O	2	0.760109186	8.4337502	5.8848429	—
23	F	22	O	2	1.847043276	6.4024324	8.4218378	—
24	F	34	O	3	0.844700813	5.0026679	7.6721678	—
25	F	21	O	2	0.652358234	11.470762	5.5325141	—
26	M	19	O	2	0.756852508	—	—	—
27	M	17	O	2	0.986197174	—	—	2.21008486
28	M	15	O	3	1.967627287	—	—	—
29	M	32	O	3	1.816722751	—	—	—
30	F	24	O	2	0.651230514	—	—	—
31	M	14	O	2	0.468885839	—	—	—
32	M	20	D	2	0.657492757	10.35606	8.8504992	—
33	M	22	D	3	0.553013682	2.8445833	15.557651	0.8475617
34	M	17	D	3	0.411514312	9.3421297	6.7479396	—
35	F	17	D	3	1.364188075	—	—	—
36	F	31	D	2	1.268633842	—	—	—
37	F	18	D	2	0.574258626	3.7410779	6.3915381	—
38	M	16	D	2	0.646167934	3.3153944	5.1450744	0.78698595
39	M	18	D	2	0.895707786	—	—	0.00215182
40	M	19	D	3	0.734310329	—	—	—
41	M	30	D	2	0.61575681	—	—	—
42	M	27	D	3	0.347842753	—	—	—

APPENDIX B
Myosin Heavy Chain Gene Expression Data

Sample	I %	IIA %	IIIX %	IIA + IIIX (%)	Neo %	Atrial %	N+A
1	46.76373	4.83346	16.3929	21.2264	13.5634	18.4465	32.00987
2	59.43631	0.7376	14.0696	14.8072	4.53356	21.2229	25.75648
3	72.63513	0.4802	1.40317	1.883365	16.9068	8.57467	25.4815
4	54.0578	4.72123	2.96599	7.687219	8.3893	29.8657	38.25498
5	82.44	0.77	1.13	1.89	3.26	12.42	15.68
6	43.19349	24.7532	8.30266	33.0559	7.42635	16.3243	23.75061
7	64.51526	1.97423	12.6901	14.66433	18.6438	2.1766	20.82041
8	—	—	—	—	—	—	—
9	19.54697	9.16923	31.4038	40.57306	8.47106	31.4089	39.87997
10	21.62082	19.122	2.47406	21.59609	8.38566	48.3974	56.78309
11	82.50461	8.50677	1.98712	10.49389	3.0848	3.9167	7.001497
12	43.67087	16.108	12.5624	28.6704	5.12386	22.5349	27.65873
13	59.47496	26.7455	3.43741	30.18291	6.0321	4.31003	10.34213
14	63.77579	5.24876	5.79756	11.04632	7.95495	17.2229	25.17789
15	68.18614	16.3654	2.94411	19.30952	4.99922	7.50512	12.50435
16	60.46	1.6	5.96	7.56	11.24	20.74	31.98
17	52.8	2.75	9.39	12.14	21.18	13.88	35.06
18	81.22045	11.0976	0.21347	11.31105	0.24352	7.22498	7.468502
19	—	—	—	—	—	—	—
20	27.15422	53.7947	3.84922	57.64394	5.23348	9.96836	15.20184
21	50.025	15.165	18.495	33.66	1.87	14.435	16.305
22	82.82	0.69	1.84	2.53	8	6.66	14.66
23	44.96595	9.55465	34.0766	43.63126	7.04012	4.36267	11.40279
24	39.735	54.325	5.295	59.62	0.0015	0.645	0.6465
25	68.84	17.43	2.31	19.74	1.64	9.78	11.42
26	71.35598	4.66844	0.63783	5.306264	13.3845	9.9533	23.33776
27	55.32481	10.3117	10.867	21.17872	12.8516	10.6449	23.49647
28	9.667862	2.45641	0.10724	2.563651	16.622	71.1464	87.76849
29	77.27445	8.50069	1.97349	10.47418	11.0351	1.21626	12.25137
30	68.98161	9.71103	3.05139	12.76242	4.32301	13.933	18.25597
31	60.59452	36.9715	1.8276	38.79906	0.25017	0.35625	0.606421
32	62.64656	1.41976	2.76143	4.181196	8.4306	24.7416	33.17225
33	56.4453	20.8024	7.55426	28.35665	2.57339	12.6247	15.19804
34	—	—	—	—	—	—	—
35	17.00384	65.0436	9.5155	74.55913	0.10384	8.33319	8.43703
36	46.48336	9.41182	3.51451	12.92633	5.48694	35.1034	40.59032
37	57.37407	20.2967	11.4907	31.78747	1.63099	9.20747	10.83846
38	32.96932	6.46314	38.0358	44.49899	4.84169	17.69	22.53169
39	56.99632	5.23804	2.81904	8.057083	21.9805	12.9661	34.9466
40	24.37489	11.8584	47.9026	59.76106	2.24507	13.619	15.86405
41	36.2	6.85	45.64	52.49	0.41	10.9	11.31
42	48.43	11.58	33.91	45.49	1.82	4.26	6.08

APPENDIX C
Masseter Muscle Fiber Percent Occupancy Data

Sample	Type I Occ	Hybrid I/II Occ	Type II Occ	Type NeoA Occ
1	55.29191483	30.7061822	10.9174551	3.084447868
2	57.04687348	18.30427916	11.31786478	13.33098258
3	52.81467722	41.47620599	1.614422254	4.094694539
4	36.87267769	38.08719342	7.813723053	17.22640583
5	82.04944642	14.66796087	0.330119513	2.952473192
6	41.86720058	54.87019872	0.537667951	2.724932753
7	24.33661707	58.35478661	9.111631123	8.196965197
8	43.5	26.1	3.5	27.00754009
9	26.00519956	23.26405491	0	50.73074553
10	29.80257325	45.94144686	5.318688495	18.9372914
11	64.38680292	27.81411058	4.677563861	3.121522636
12	29.86687756	45.68750005	0.328707269	24.11691512
13	—	—	—	—
14	69.34743653	21.21650395	0	9.436059527
15	46.16011358	39.19839353	0.545175493	14.0963174
16	52.48494995	23.64652457	0.33227345	23.53625204
17	42.66587712	43.92100935	3.189911015	10.22320251
18	79.98243138	17.81806887	2.157292333	0.04220742
19	31.05278916	44.21556203	0.451174305	24.28047451
20	36.43366551	58.87250378	2.469895203	2.223935503
21	59.88668147	13.07228697	4.895627969	22.14540359
22	63.77991078	29.27552458	0.824101513	6.120463123
23	65.41912492	6.45601295	20.19907985	7.925782276
24	28.17971237	1.9256878	69.89459983	0
25	74.04128636	24.78100704	0.154818548	1.022888061
26	61.30819713	34.20832382	0.561826071	3.921652981
27	79.20276989	12.16880476	2.387054245	6.241371104
28	50.11661306	26.86720004	0.073613029	22.94257387
29	27.34604354	68.25740662	3.809010187	0.587539656
30	49.21476989	48.41176783	1.80044309	0.573019189
31	—	—	—	—
32	39.10759357	40.27640339	10.95286647	9.663136567
33	34.27065469	48.48685901	17.24248631	0
34	25.25153378	47.73235157	21.23515245	5.780962196
35	18.07232041	14.5664479	60.33999343	7.021238254
36	36.91918769	45.4925912	13.72160485	3.866616272
37	66.78451377	1.86028594	31.35520029	0
38	42.23599165	16.21084165	34.1419126	7.41125409
39	—	—	—	—
40	60.75137256	8.608271589	24.69208657	5.948269277
41	45.5472213	4.672650939	37.26409847	12.5160293
42	50.87917031	5.209638472	40.30981645	3.601374765

APPENDIX D
MYO1C Expression, Myosin Heavy Chain Gene Expression
& Fiber Percent Occupancy in Limb Samples

<u>Muscle</u>	<u>MYO1C</u>	<u>I %</u>	<u>IIA %</u>	<u>IIIX %</u>	<u>IIA + IIIX %</u>	<u>Neo %</u>	<u>Atrial %</u>	<u>N+A%</u>
Forearm Extensor+ Trapezius	0.900418 22	46.12 452	44.501 65	9.0322 3	53.5338 8203	0.0005 6	0.341033 088	0.34159 31
Triceps/ Deltoid	0.995123 33	59.84 482	29.456 15	10.206 71	39.6628 6624	0.0011 029	0.491210 724	0.49231 36
Triceps	0.779978 1	45.23 221	52.994 76	2.1725 77	55.1673 3704	0.0082 026	0.189679 619	0.19788 22
Quadri- ceps	0.782778 68	51.62 975	30.686 28	16.995 33	47.6816 1396	0.4047 611	0.283870 265	0.68863 13
Avg	0.864574 58	50.70 783	39.409 71	9.6017 13	49.0114 2482	0.1036 566	0.326448 424	0.43010 51
n	4	4	4	4	4	4	4	4
StD	0.103561 38	6.716 24	11.338 03	6.0703 65	7.01217 5575	0.2007 665	0.126330 168	0.21013 29
SEM	0.051780 69	3.358 12	5.6690 16	3.0351 82	3.50608 7787	0.1003 832	0.063165 084	0.10506 65

<u>Muscle</u>	<u>Type 1 Occ</u>	<u>Hybrid I/II Occ</u>	<u>Type IIA Occ</u>	<u>Type IIIX Occ</u>	<u>Type Neo Occ</u>	<u>Type Atrial Occ</u>	<u>Type NeoA Occ</u>
Forearm Extensor+ Trapezius	30.83560 5	0	66.2177715	2.946623 8	0	—	0
Triceps/ Deltoid	47.96853 6	0.0292003	42.715016	9.287247 3	0	—	0
Triceps	39.62943 7	0	49.975185	10.39537 8	0	—	0
Quadri- ceps	36.95035 6	0.9930873	62.05657	0	0	—	0
Avg	38.8459 835	0.255571 9	55.241135 63	5.65731 2275	0	—	0
n	4	4	4	4	4	—	4
StD	7.10854 7222	0.491869 583	10.825442 1	4.99929 0259	0	—	0
SEM	3.55427 3611	0.245934 791	5.4127210 48	2.49964 513	0	—	0

APPENDIX E
Correlation Values of MYO1C to Myosin Heavy Chain Gene
Expression and Fiber Percent Occupancy

APPENDIX E1

Group	I	IIA	IIX	IIA/IIX	Neo	Atrial	NA
D2 (N=5)	-6%	-33%	-33%	-33%	60%	<u>73%</u>	<u>100%</u>
D3 (N=4)	-66%	66%	0%	66%	-33%	33%	33%
N2 (N=6)	<u>-86%</u>	60%	46%	60%	-6%	<u>73%</u>	<u>73%</u>
N3 (N=4)	0%	20%	20%	0%	60%	-40%	0%
O2 (N=10)	-1%	-9%	20%	1%	-1%	-1%	-1%
O3 (N=6)	-6%	-46%	-60%	<u>-73%</u>	<u>73%</u>	20%	20%
Total (N=35)	0%	5%	-9%	-3%	18%	4%	13%

Significant correlations are bolded and underlined ($p < 0.05$)

APPENDIX E2

Group	Type 1 Occ	Hybrid I/II Occ	Type II Occ	Type NeoA Occ
D2 (N=5)	<u>-100%</u>	<u>100%</u>	-40%	0%
D3 (N=4)	-20%	20%	20%	66%
N2 (N=6)	<u>-80%</u>	23%	4%	33%
N3 (N=4)	-66%	<u>100%</u>	0%	0%
O2 (N=10)	37%	<u>-55%</u>	15%	20%
O3 (N=6)	-4%	4%	-14%	-33%
Total (N=35)	-12%	9%	-7%	8%

Significant correlations are bolded and underlined ($p < 0.05$)

APPENDIX F
Correlation Values for MYO1C to IGF-1 and MSTN

<u>Sample</u>	<u>Sex</u>	<u>Age</u>	<u>Vert</u>	<u>Sagitt</u>	<u>MYO1C RQ</u>	<u>IGF1 RQ</u>	<u>GDF8 RQ</u>
1	F	25	N	2	0.450896293	3.3698769	4.1672816
3	F	13	N	2	0.399792761	6.9832234	6.4226942
5	M	16	N	2	0.260094643	4.8803782	6.488358
6	F	15	N	2	0.744078636	2.9228557	4.8453128
9	M	29	N	2	0.747142375	8.2712021	16.844664
					Correlation=	0.0911734	0.4778573
4	F	15	N	3	1.128490806	4.8912811	3.3111749
2	M	17	N	3	0.413070679	2.8790464	4.966886
					Correlation=	1	-1
14	M	18	O	3	0.999677896	7.2780008	13.056429
15	F	16	O	3	0.577373743	6.6750426	6.8897848
19	F	15	O	3	0.48182857	3.428965	4.669999
21	F	32	O	3	0.534011722	3.3037183	5.8248711
24	F	34	O	3	0.844700813	5.0026679	7.6721678
					Correlation=	0.6820554	0.9200251
16	F	18	O	2	1.009588838	7.239387	2.8055847
17	F	19	O	2	0.617408693	9.2330894	5.828546
18	F	20	O	2	1.249668956	7.7892556	2.5832822
22	F	18	O	2	0.760109186	8.4337502	5.8848429
23	F	22	O	2	1.847043276	6.4024324	8.4218378
25	F	21	O	2	0.652358234	11.470762	5.5325141
					Correlation=	-0.7791796	0.299085
33	M	22	D	3	0.553013682	2.8445833	15.557651
34	M	17	D	3	0.411514312	9.3421297	6.7479396
					Correlation=	-1	1
37	F	18	D	2	0.574258626	3.7410779	6.3915381
38	M	16	D	2	0.646167934	3.3153944	5.1450744
32	M	20	D	2	0.657492757	10.35606	8.8504992
					Correlation=	0.5608595	0.3074255

APPENDIX G
Correlation Values for MYO1C and MYO1H to Fiber Type

	MYO1C	MYO1H
I%	0.2051813	-0.1523005
IIA%	-0.0133931	-0.0105544
IIIX%	-0.3022634	0.3237343
IIA/IIIX %	-0.2501329	0.2536645
Neo %	0.1828944	-0.1755943
Atrial %	-0.2235251	-0.0024796
N+A%	0.0369029	-0.1363647
Type I Occ	0.2587681	0.1673905
Hybrid I/II Occ	-0.1399645	-0.4505026
Type II Occ	-0.3016096	-0.0135977
Type NeoA Occ	0.1072984	0.4713478