DIFFERENTIAL EXPRESSION OF CALSARCIN GENES IN ORTHOGNATHIC SURGERY PATIETNS WITH *ACTN3* R577X GENE DEVIATIONS

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ABSTRACT

Objective: Malocclusion is a complex musculoskeletal trait, with muscle playing an integral role in vertical facial development. A single nucleotide polymorphism (SNP) produces the R577XX nonsense mutation in the alpha-actinin-3 (*ACTN3*) gene, creating a stop codon and loss of its protein. With loss of ACTN3, alpha-actinin-2 (*ACTN2*) is upregulated. Calsarcins, known inhibitors of calcineurin activation, preferentially bind *ACTN2* leading to a surge in free calcineurin. The increase in calcineurin activity produces the phenotypic shift of fast muscle fibers toward the slow myogenic program seen in the *ACTN3* null genotype (Seto et al., 2013). Here, we have tested whether calsarcin gene expression is affected by *ACTN3* genotypes in human masseter muscle.

Methods: Subjects undergoing orthodontic treatment and orthognathic surgery were recruited from the University of Lille, Department of Oral and Maxillofacial Surgery in Northern France. During the bilateral sagittal split osteotomy, masseter muscle samples were collected from the discarded section of deep anterior superficial masseter muscle, snap frozen, and shipped to Dr. Sciote's lab at Temple University.

RNA from masseter muscle samples was isolated from 41 subjects using TRIzol^{TM} reagent. *MYOZ* gene expression was quantified by RT-PCR using an adult skeletal muscle reference standard (commercially prepared skeletal muscle RNA; Ambion, Inc), and individual primer-probe sets for *MYOZ1*, *MYOZ2*, *MYOZ3*, and *HPRT1* (utilized for normalization of data).

ANOVA and unpaired t-tests were used to determine the significance of expression differences between *MYOZ* genes and by *ACTN3* R577X genotypes, as well as by malocclusion

classes. Pearson analyses were used to determine correlations between *MYOZ* expression and fiber type mean percent occupancies.

Results: The main aim of this project was to determine whether expression of the three calsarcin genes, *MYOZ*1, 2, and 3, differs between subjects with RR, RX and XX genotypes for the *ACTN3* gene, as well as between sagittal and vertical classes of malocclusion, asymmetries and TMD. Differences were found for *MYOZ3* expression where relative quantities in males, but not females, decreased progressively from the *ACTN3* RR, to RX, and XX genotypes. Among subjects with the RX genotype, expression differed significantly between males and females by an unpaired *t*-test. A statistically significant difference was detected between *MYOZ*2 and Class II, Class III malocclusions (p=0.05). Sagittal differences were compared further by ANOVA analyses with a statistically significant difference detected for *MYOZ*3 with a probability of 0.02.

Correlation analyses comparing fiber type mean % occupancy with calsarcin gene expression revealed a significant positive relationship between *MYOZ2* and type I (slow-twitch) fibers. Correspondingly, a significant correlation of *MYOZ2* expression with type IIA and IIX (fast-twitch) fibers was negative.

Conclusions: The greatest relative quantity of RNA for the three calsarcin genes was found in *MYOZ*3, suggesting more calsarcin-3 may be needed in masticatory muscle structure and function than other calsarcin isoforms. Alternatively, high expression of *MYOZ3* in the masseter samples may indicate that there are relatively greater amounts of that isoform in cranial muscle than in the limb skeletal muscle standard used in these studies. Also, relative quantities of *MYOZ*3 expression in males decreased progressively from the *ACTN3* RR, to RX, and XX genotypes. While this data may suggest that the ACTN3 R577X polymorphism may affect MYOZ3 expression in males of the malocclusion patient population, an increased sample of male subjects would be needed to determine if this trend has true significance. Expression of *MYOZ2* (calsarcin-1) was strongly correlated with slow fiber-type occupancy in masseter muscle of our patient population. The muscle-specific expression of each calsarcin may lend to the understanding of this result. MYOZ2 is the only isoform found in both cardiac muscle and slow-twitch skeletal muscle, while MYOZ1 and MYOZ3 are both found in skeletal muscle with a predilection towards fast-twitch skeletal muscle (Frey et al., 2004).

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CHAPTER 1

INTRODUCTION

Abnormalities in the skeletal development of the jaw are not solely responsible for malocclusion. Instead, malocclusion is a complex musculoskeletal trait, with skeletal muscle playing a key environmental role in variations of overall vertical facial development. Masticatory muscle strength, determined by the size and proportion of muscle fiber types, corresponds with various vertical facial deformations. For example, masticatory muscle of individuals with deep bite malocclusion has increased size and overall area of type-II fast-contracting fibers (Sciote et al., 2012).

Genome wide association studies (GWAS) of muscle size and strength demonstrate that skeletal muscle fiber type properties are influenced by genetic variations. The most common of these variations includes single nucleotide polymorphisms (SNPs) within gene sequences. Alpha-actinin-3 (*ACTN3*) is a gene in which one such SNP, R577X (rs1815739), produces a stop codon and loss of its protein product, yet individuals carrying the mutation are seemingly unaffected (Seto et al., 2013).

In a study of phenotypic effects of *ACTN3* gene deviations in a population of orthognathic surgery patients, subjects with the R577XX genotype were compared to both the heterozygote 577RX and homozygote 577RR genotypes (Zebrick et al., 2014). Both vertical and sagittal discrepancies present within each patient were related to the masseter fiber type properties. Patients with the 577XX genotype were underrepresented in the deep bite malocclusion population, which supports evidence that vertical facial dimensions are affected by concordance between presence of fast-contracting fibers and *ACTN3* gene activity in masticatory muscle. In contrast, a greater incidence of 577XX in skeletal Class II subjects is accompanied by smaller fast type II fibers and probable biologic influence on bone growth.

Alpha-actinin-2 and -3 are found within the Z-disk of the skeletal muscle sarcomere where they influence fiber-type properties. Also found in the Z-disc is a family of 3 muscle-specific signaling proteins known as calsarcins. Actinins bind the calsarcins to alter the presence of the calcium-dependent, calmodulin-stimulated protein phosphatase, calcineurin, which is a modulator of signal transduction. With the *ACTN3* R577XX genotype, lack of ACTN3 results in increased free calcineurin through the preferential binding of calsarcin-2 with alpha-actinin-2. Muscle fiber-type properties are then affected by the increase in free calcineurin activating a slow myogenic fiber program (Wang et al., 2006). In this study, it will be determined whether the *ACTN3* genotypes influence the expression of calsarcin family proteins in human masseter muscle.

CHAPTER 2

REVIEW OF THE LITERATURE

2.1 Genetics of Malocclusion

The influence of genes on the development of orofacial structures suggests the importance of studying genetic factors in the etiology of malocclusion. Craniofacial development is not just directed by genetic factors, but rather their interactions with the environment. Therefore, the best evidence for distinguishing the specific contributions of genetics and the environment comes from family and twin studies (Mossey, 1999).

A significant deviation from normal or ideal occlusion defines a malocclusion (Andrews, 1972). Normal occlusion is dependent on many components within the craniofacial complex, such as the size of the maxilla and mandible, cranial base relationships, arch form, and the size and number of teeth (Mossey, 1999). The presence of malocclusion is almost non-existent in populations such as early Pacific islanders that are genetically homogeneous. However, heterogeneous populations present with a significantly greater incidence of jaw discrepancies. In 1941, experiments on the crossbreeding of dogs resulted in dramatic malocclusions indicating that the major cause of malocclusion in heterogeneous populations is due to the independent inheritance of tooth and jaw characteristics (Stockard, 1941). The validity of these experiments has since been disproven due to the presence of the gene for achondroplasia and its variable expressivity in dogs. Likewise, familial studies provide a more credible way to determine the extent with which a characteristic is determined by inheritance.

The underlying principle of twin studies is to observe differences between monozygotic and dizygotic twins. Monozygotic twins are the result of a single fertilized egg splitting. Therefore, monozygotic twins share the same chromosomal DNA, making them genetically identical. Any difference's detected between monozygotic twins are due solely to environment. Dizygotic twins share 50% of their total gene complement, just as ordinary siblings, meaning both environment and genotype result in differences between them (Proffit, 2013). In a 1961 study of identical and fraternal twins, highly significant hereditary variations were found in the anterior cranial base, mandibular body length, lower face height, and total face height with linear cephalometric measurements (Horowitz et al, 1961). With numerous familial studies, evidence supports that the craniofacial structure and the potential for various types of malocclusion is strongly impacted by the person's genotype.

Familial occurrence of malocclusion is best demonstrated by a Class III malocclusion consistently appearing within the Hapsburg royal family lineage. By constructing and analyzing a detailed pedigree, it was determined that mandibular prognathism occurred by a single autosomal dominant gene with high penetrance (Wolff et al, 1993). Additional studies suggest mandibular prognathism occurs by polygenic inheritance. In a 1965 study of mandibular prognathism in monozygotic and dizygotic twins, Schulze and Weise determined that concordance was six times greater in monozygotic twins. In recent years, various modes of inheritance and a wide range of environmental factors have been suggested as key factors in the development of not just mandibular prognathism, but also all existing malocclusions.

Genetic and environmental factors influence the development of craniofacial structures. Thus, understanding the underlying genetic factors in development and evidence of how their activity is influenced by environmental changes is imperative to determining the etiology of malocclusion (Calkan et al., 2012).

2.2. Skeletal Muscle Functioning

Craniofacial skeletal morphology is also influenced by masticatory muscle fiber type properties and function. The complex relationship between jaw growth and muscle architecture requires an understanding of the intrinsic composition of muscle in terms of its fiber types (Rowlerson, 2005).

Three main muscle fiber groups have been established for human skeletal muscle by myofibrillar ATPase activity: type I (slow-twitch), IIA and IIX (fast twitch). While human skeletal muscle is mostly limited to the three fiber groups, human masseter muscle contains highly specialized muscle fibers leading to the identification of eight fiber types. This includes type I, IIA, and IIX in addition to type IM, IIC, IIX fibers and type neonatal and alpha-cardiac (Sciote et al., 1994; Sciote et al., 2003). As well as demonstrating an increased number of fiber types, human masseter muscle displays a wide variation in fiber-type composition between individuals (Rowlerson et al., 2005).

In a study comparing muscle fiber type properties among subjects with open bite, deep bite, and normal malocclusions, significant differences were detected amongst the varying vertical dimensions, but not within the sagittal Class II or III groups. Therefore, fiber type properties, such as fiber type size and tissue percent occupancy, are related to variations in the vertical development of the face. An increase in vertical facial development is inversely related to an increase in type II mean fiber areas and percent tissue occupancies. Thus, a decrease in facial height would occur as the number of type II fibers and their area increased (Sciote et al., 2012). This increase in type II fibers in deep bite individuals is in accordance with increases in average bite force. In normal vertical dimension, approximately half of the tissue volume is composed of type I fibers. Type I fibers have the largest mean area and are often the most abundant. Type II fibers have substantially smaller mean areas in masseter muscle than type I fibers and a resultant decreased force production. Open bite individuals display a fiber type composition similar to that of normal individuals, except for decreased type II fibers (Rowlerson et al., 2005). Thus, type I fiber proportions, but not variations in fiber size, exert moderate influences vertical facial growth. In contrast, it is the size and percent tissue occupancy of type II fibers, which is the most significant (Sciote et al., 2012).

2.3. The Paradigm Shift

With current understanding of the influence of masseter muscle fiber type properties on bone deformation, the long held model assuming that human malocclusions are solely the result of skeletal development must re-evaluated. This paradigm shift recognizes that malocclusion is a complex musculoskeletal trait affected by combinations of transcription and growth factors acting on bone, teeth, and skeletal muscles. Therefore, an efficient way of identifying genes that influence abnormal craniofacial morphology would be to target those known to effect musculoskeletal growth and function (Sciote et al., 2013).

Genome wide association studies (GWAS) of muscle size and strength have demonstrated that both muscle size and height are highly heritable anthropometric traits. Genetic variations, most often single nucleotide polymorphisms (SNPs) within gene sequences with functional consequences, have been shown to influence skeletal muscle fiber type properties (Sciote et al., 2013). Alpha-actinin-3 (*ACTN3*) is one such gene of interest with its influence on fiber type proportions and muscle performance (Vincent et al., 2007).

2.4. The *ACTN3* Null Genotype

Through the work of Kathryn N. North, enhanced endurance activity was discovered in a small subset of humans lacking α-actinin-3. As stated previously, loss of α-actinin-3 results from homozygosity for a common nonsense polymorphism in the *ACTN3* gene. The *ACTN3* 577XX-null genotype is highly represented in elite endurance athletes, while lacking in elite sprint and power athletes. This heightened endurance capacity was further demonstrated in *ACTN3* knockout mice, thus mimicking the phenotypic effects found in humans.

The molecular basis for the effects of this nonsense mutation relate to increased calcineurin activity. With the absence of α-actinin-3, α-actinin-2 is upregulated more than two-fold that of control mice to compensate for the loss of the α-3 isoform. In contrast, expression of calsarcin-2, a known inhibitor of calcineurin activation, does not differ between *ACTN3* knockout mice and controls. Therefore, the increased calcineurin activity could not be accounted for by differences in calsarcin quantities. It was instead demonstrated that calsarcin-2 preferentially binds α-actinin-2 over α-actinin-3. This enhanced binding affinity, as illustrated in **Figure 1**, in addition to the competitive binding of α-actinin-2 and calcineurin for calsarcin-2, leads to the increased release of calcineurin from calsarcin inhibition. The increase in free calcineurin produces the phenotypic shift of fast muscle fibers toward the slow myogenic program seen in *ACTN3* null genotype (Seto et al., 2013).

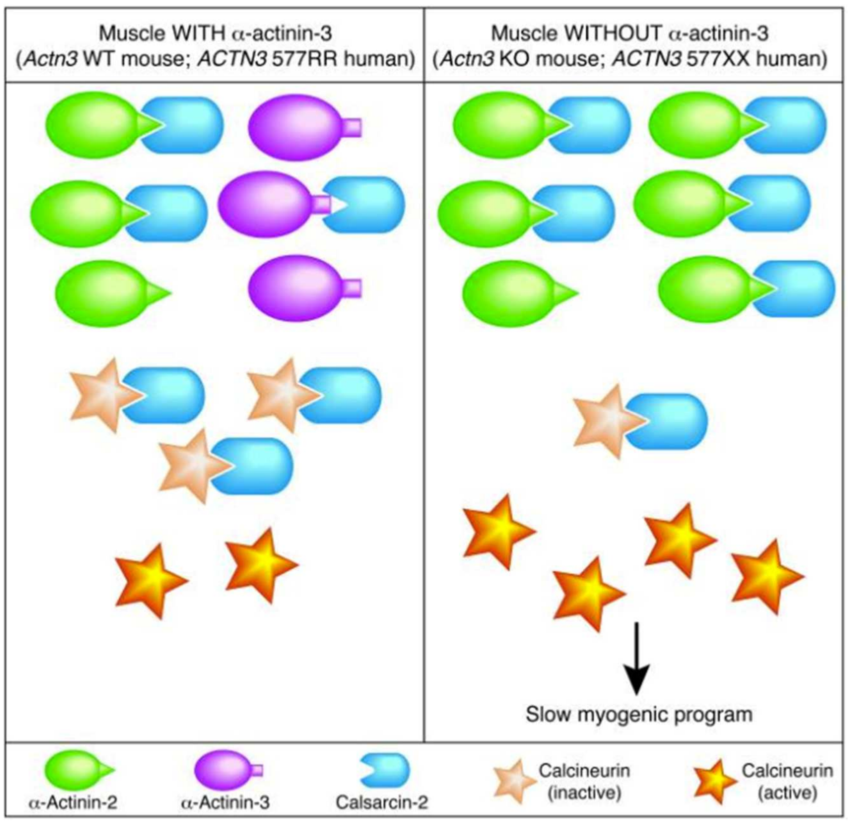


Figure 1. Schematic of Sarcomeric α-Actinin Regulation of Calcineurin. α-Actinin-2 is differentially expressed in muscles without α-actinin-3 (Replicated from Seto et al., 2013).

2.5. Malocclusions and the *ACTN3* R577X Genotypes

The occurrence of the *ACTN3* 577XX genotype in orthognathic surgery patients and expression of *ACTN3* in the masseter were studied to associations with malocclusions. Masseter muscle biopsies and saliva samples were obtained from the sample population for the expression and *ACTN3* single nucleotide polymorphism genotyping studies. Histomorphometric analysis was performed to identify the fiber type of masseter muscle samples, while real-time polymerase chain reaction (RT-PCR) quantified the mRNA expression between subjects (Zebrick et al., 2014).

The results of these studies were used to compare differences between R577X polymorphism genotypes and corresponding phenotypes to assess whether loss of α-actinin-3 affects fiber-type, muscle metabolism, and bone mineralization associated with states of malocclusion. The *ACTN3* genotype frequency varied between the defined sagittal and vertical malocclusion classifications. In skeletal Class II surgery patients, masseter muscle exhibited an elevated R577XX genotype. Patients classified with a deepbite malocclusion displayed a lack of the R577XX genotype. Significantly smaller type II fiber diameters in masseter muscles were found with α-actinin-3 loss. While Kathyrn North's study showed an upregulation of α-actinin-2 in ACTN3 knockout mice, RT-PCR revealed unchanged *ACTN2* expression levels in masseter muscles (Seto et al., 2013; Zebrick et al., 2014).

2.6. Sarcomeric Structure and the Z-Disc

The sarcomere represents the basic contractile unit of striated muscle cells. It is a repeating structure delineated by a Z-disc border. Sarcomeres are approximately 2 µm long and 3 µm wide. They connect end-to-end to form a long, thin strand known as a myofibril. Multiple myofibrils align in a parallel fashion to create a muscle fiber. Thus, sarcomeres are the building blocks from which all striated muscles, including cardiac, are created.

The pattern created by the alternating ordered arrays of actin and myosin filaments within a sarcomere lends itself to the name striated muscle. The central A-band is created by thick myosin filaments. Proteins at the center M-band cross-link with adjacent myosin filaments, forming the M-region. Thin actin filaments are attached to the Z-bands at the end of each sarcomere. They partially overlap with myosin filaments in the A-band. The I-band is the region consisting solely of actin filaments, whereas the H-zone represents the area occupied by only myosin filaments (Squire, 1997).

The Z-disc serves as the interface between the cytoskeleton and the contractile unit. A major component of the Z-disc is α-actinin, to which actin-based thin filaments are cross-linked with in an anti-parallel fashion. This organization provides the means for transmission of force longitudinally. The contractile mechanism is initiated by a series of molecular movements within the sarcomere. Without muscle stimulus, myosin-binding sites on actin are capped by tropomyosin. Sites are exposed when nervous stimuli cause the release of calcium ions into the myofibril. A calcium ion binds to troponin, which alters the configuration of tropomyosin and subsequently allows attachment of myosin heads to actin. The hydrolysis of ATP into inorganic phosphate and ADP on myosin facilitates attachment of the myosin head to actin. Release of myosin from actin occurs when a new ATP molecule binds to the myosin head. Therefore, muscle contraction involves an intricate system of coordinated steps.

In addition to its mechanical role in force transmission, the Z-disc is a focal point for the sensing and transmission of important internal and external signaling pathways, due to its close proximity to the plasma membrane, as well as its associated ion channels and receptors (Frank, et al. 2006; Frey et al., 2002; Squire, 1997; Wang et al., 2007).

2.7. Z-Disc Proteins

An increasing number of Z-disc proteins have been identified since the realization of the Z-disc's role in important signaling pathways. A protein is identified specifically as a Z-disc protein when biochemical evidence of a direct protein-protein interaction with an established Z-disc protein is found by electron microscopy. Due to their central involvement in the structure and diverse functions of the Z-disc, Z-disc protein mutations have been implicated in a wide range of muscular diseases from cardiomyopathies to muscular dystrophies.

As mentioned previously, α-actinin is a major protein component of the Z-disc. There are four members of the α-actinin family, with α-actinin-1 and α-actinin-4 only found in non-muscle tissues. α-actinin-2 and α-actinin-3 are specifically striated-muscle-isoforms, with α-actinin-2 serving as the only cardiac isoform. α-actinin-3 has been recently identified as a functionally dispensable isoform (Frank et al., 2006). Kathryn N. North identified a common nonsense mutation that resulted in an α-actinin-3 deficiency in approximately 16% of the general population. This deficiency serves as a rare example in which health was maintained after mutational loss of a protein. Human disease predominately occurs with α-actinin mutations due to its diverse protein interactions and functions (Seto et al., 2013).

γ-filamin is the filamin isoform specific to striated muscle. Filamin resembles actinin in their cross-linking properties and actin-binding. γ-filamin has several binding partners within the Z-disc. This includes myotilin, a limb girdle muscle dystrophy-associated Z-disc protein, as well as the calsarcin protein family at the C-terminus end of γ-filamin. Because of its associations with other Z-disc components, γ-filamin is also implicated as a candidate gene for muscle myopathies.

T-Cap/telethonin is a small Z-disc protein that is associated with the structure and signaling of the Z-disc. It is involved in the negative regulation of calcineurin and hypertrophic signaling through its interaction with the potassium channel subunit minK/isk. T-Cap also associates with the calsarcin protein family and prevents the release of myostatin, a negative regulator of skeletal muscle mass.

Myotilin is a Z-disc protein involved in signaling. It co-localizes with α-actinin-2 at the Z-disc. It has also been shown to interact with γ-filamin, actin, as well as calsarcin-1 and calsarcin-2. While the functional relevance of these protein interactions remain unclear, it is important to note that mutations in the myotilin gene are associated with muscle diseases referred to as myotilinopathies (Frank et al., 2006).

Calcineurin is a calcium-calmodulin-dependent signal transducer in the sarcolemma of striated muscle cells. While the transduction mechanism of calcineurin has been studied most comprehensively in T lymphocytes, the properties of striated muscle cells have recently been shown to be profoundly influenced by this transducer. In cardiac muscle, cardiac hypertrophy, followed by heart failure and sudden death in transgenic mice was induced by the overexpression of activated calcineurin by hypertrophic agents and alterations in sarcomere length. Calcineurin also promotes the slow-twitch phenotype in skeletal muscle through differentiation and fiber-type specialization following its activation (Frey et al., 2002). In conducting a yeast two-hybrid screen with a calcineurin subunit as bait, Frey, Richardson, and Olson discovered a new striated-muscle specific family of Z-disc proteins they termed calsarcins (Frey et al., 2000).

Calsarcins bind calcineurin to the sarcomere of cardiac and skeletal muscle by binding to the Z-disc protein α-actinin. Hence, the name calsarcin was formulated for these calcineurin-associated sarcomeric proteins. In addition to interactions with calcineurin, calsarcins were also proven to interact with γ-filamin, T-Cap/telethonin, and myotilin by co-immunoprecipitation. While mutations in the calsarcin genes have not been linked to human disease, the aforementioned calsarcin-associated proteins have been proven to cause cardiomyopathies and muscular dystrophies. This would indicate that calsarcins are central to human myopathies and may be potentially identified as candidate genes themselves (Frey et al., 2006).

2.8. The Calsarcin Family

The calsarcin family is made up of three isoforms, calsarcin-1, -2, and -3. Calsarcin-1 is encoded by the myozenin 2 (*Myoz2*) gene, while calsarcin-2 and calsarcin-3 are represented by the myozenin 1(*Myoz1*) gene and myozenin 3 (*Myoz3*) gene, respectively. Through Northern Blot analysis of human and mouse tissues, the muscle-specific expression of each calsarcin was determined. Calsarcin-1 is expressed in cardiac and skeletal muscle fibers, specifically types I and IIa. Calsarcin-2 is solely located in adult and mouse skeletal muscle, predominately fast-twitch fibers. The final member of the calsarcin family, calsarcin-3, is also found in skeletal muscle, with a predilection towards fast-twitch muscle fibers. Therefore, calsarcin-1 is the only isoform found in both cardiac muscle and slow-twitch skeletal muscle. Calsarcin-2 and -3 are each expressed in fast-twitch skeletal muscle.

The fiber-type specificity of calsarcin-1 and -2 expressions in skeletal muscle was determined by *in situ* hybridization. Sections of adult mouse hindlimb and calsarcin-1 and -2 probes were used. Soleus and plantaris muscles, which are predominately composed of slow-twitch fibers, exhibited expression of calsarcin-1. In contrast to calsarcin-1, calsarcin-2 was localized to gastrocnemius, which is predominantly a fast-twitch muscle type. These results correspond with the muscle-specific expression of each calsarcin isoform, as determined by the Northern Blot analysis.

Calsarcin's role in signal transduction was first indicated by the co-localization of calsarcin and calcineurin at the Z-disc and further supported by its interaction with multiple Z-disc proteins. To determine the function of calsarcin-1 *in vivo*, a mouse knockout model for calsarcin-1 was created. The fiber-type composition was then analyzed in the mice lacking calsarcin-1. The studies indicated a significant increase in the number of type I (slow) fibers within soleus muscle, consistent with calcineurin activation. A significant surge in the activity of calcineurin was also directly measured in the soleus muscle of the calsarcin-1-null mice. A hypertrophic gene program was also activated by the absence of calsarcin-1. Cardiac hypertrophy did not occur in unstressed null mice, but in response to calcineurin activation or pressure overload, exaggerated pathological hypertrophy did develop. These results suggest that calsarcin-1 is responsible for the regulation of calcineurin signaling in striated muscle, ultimately influencing the heart's response to biochemical stress and skeletal muscle fiber type (Frey et al., 2004).

The function of calsarcins in fast-twitch skeletal muscle *in vivo* was also determined by the creation of a mouse knockout model of calsarcin-2. Calsarcin-2 deficient mice versus wild-type controls had significantly decreased body weights and reduced fast-twitch muscle masses without the presence of any obvious myopathy. *MYOZ1* knockout mice exhibited significantly improved running capabilities and performance in exercise studies. Fiber-type analysis of calsarcin-2 deficient mice revealed a switch towards slow-twitch, oxidative fibers, specifically type IIA fibers in fast-twitch muscles. These findings are consistent with the increase in calcineurin activity displayed in knockout mice as compared to their matched controls. As shown in calsarcin-1 deficient mice, calsarcin-2 also negatively regulates the activity of calcineurin. By eliminating calsarcin-2, calcineurin activity is no longer inhibited, promoting the transformation to slow-twitch oxidative fibers and ultimately, enhanced endurance capacity (Frey et al., 2008).

2.9. Selection of Genes of Interest

The intimate association of calsarcins and calcineurin with Z-disc α-actinin proteins has been studied extensively. These associations were further elucidated for ACTN3 R577X genotypes by North and Frey. Accordingly, observations from these studies, along with the report from Zebrick et al. on masseter muscle, were fundamental to an investigation of the role of calsarcins in masseter muscles of orthognathic surgery patients with different genotypes for the ACTN3 polymorphism.

CHAPTER 3

AIMS OF THE INVESTIGATION

In this study, expression of the calsarcin family of genes will be determined in a population of orthognathic surgery patients representing different classes of malocclusion in both vertical and sagittal dimensions and categories of genotype for the *ACTN3* R577X polymorphism. The specific aims include:

Aim 1: To determine whether expression of the three calsarcin genes differs between subjects with RR, RX and XX genotypes for the *ACTN3* gene.

Aim 2: To test whether differences in calsarcin gene expression occur between classes of malocclusion.

Aim 3: To determine correlation values between calsarcin gene expression and fiber-type properties in masseter muscles of malocclusion subjects.

CHAPTER 4

MATERIALS & METHODS

4.1 Study Subjects

Study subjects were recruited from the University of Lille, Department of Oral and Maxillofacial Surgery in Northern France. All individuals were undergoing orthodontic treatment and orthognathic surgery for the correction of dentofacial malocclusions. Informed consent was obtained from all participants at the time of treatment. Approval was obtained from the French Independent Ethical Committee and the Institutional Review Board at Temple University for conduct of this research protocol. Delaire's analysis was used to determine the pre-surgical diagnoses of all participants (Delaire, 1981). The vertical and sagittal jaw movements to be made in the bilateral sagittal split osteotomy surgery further confirmed diagnoses. Subjects were diagnosed with a sagittal Class 1, 2, or 3 jaw relationship and an open, deep, or normal vertical skeletal jaw malocclusion. Saliva samples were collected from each subject for genotype analysis by Dr. Alexandre Vieira of the University of Pittsburgh to identify those having normal CC, heterozygous TC, or null TT *ACTN3* genotypes. During the bilateral sagittal split osteotomy, samples were collected from the discarded section of deep anterior superficial masseter muscle. Samples were snap frozen in liquid nitrogen and stored at -80°C in Lille before shipment to Dr. Sciote's lab at Temple University as described by Rowlerson et al. (2005).

In Dr. Sciote's laboratory, muscle samples were analyzed for fiber-type composition and RNA was isolated for determination of gene expression by reverse-transcriptase real time polymerase chain reaction (RT-PCR).

4.2 Immunohistochemistry of Masseter Muscle

Masseter muscle composition was determined by first serially sectioning frozen muscle samples into 10μm thick slices. Sections were then mounted on glass slides and surveyed for immunohistochemical fiber types using antibodies specific for myosin heavy chain (MyHC) isoforms (Rowlerson et al., 2005). Anti-type I, anti-type-II, anti-type IIA, anti-type-neonatal, and anti-α-cardiac (atrial) antibodies were used. Four fiber-type groups were classified following analysis, as type I (slow contracting, fatigue resistant), type hybrid (combinations of slow and fast contractile properties), type II (fast contracting, either fatigueable or fatigue resistant), and type neonatal-atrial. Image J image-analysis software type-classified and measured the cross-sectional areas of all fibers within selected tissue sections.

4.3 Genotyping

Saliva samples were collected from consenting orthognathic surgery patients with Oragene® DNA Self-Collection kits. DNA was extracted and genotyping performed on all samples by Dr. Alexandre Vieira of the University of Pittsburgh. *ACTN3* genotype frequencies were compared at two single nucleotide polymorphisms (SNPs), rs1815739 (the R577X polymorphism) and rs678390 (intron area with no known functional consequences). The first SNP is a cytosine to thymine transition that produces a stop codon at residue 577. This results in three genotypes CC or RR (normal), TC or RX (heterozygote), and TT or XX (no α-actinin-3 produced). The second SNP is a Cytosine to Thymine transition within an *ACTN3* gene intron, therefore producing no functional consequences. Three genotypes are produced, designated here as CC (normal), TC, and TT. Along with Lille surgery subjects, 31 additional control subjects were selected from the genotyping data bank DNA Repository at the University of Pittsburgh.

4.4 Quantification of Calsarcin mRNA

Samples from a total of 41 subjects were utilized, 15 of which were males and 26 females. Of the 26 women studied, 20 had Class II malocclusions, while 6 had Class III malocclusions. Vertical occlusion in the Class II group included 10 open bite patients, 5 deep bite patients, and 5 with a normal bite. The Class III group included no subjects with normal vertical occlusions, 3 with deep bite, and 3 with open bite. 15 samples from men were utilized, of which 8 had Class II malocclusions and 7 had Class III malocclusions. The Class III group included 3 men with normal vertical occlusions, 1 with deep bite, and 3 with open bite. Men within the Class II group included 5 with normal vertical occlusions, 1 with deep bite malocclusion, and 2 with open bite malocclusions.

RNA from masseter muscle samples was isolated with TRIzol™ reagent, digested with 10 U DNase, and then heated to 65 ° C to inactivate the DNase. RNAqueous® (Ambion, Austin, Texas) was used to re-isolate and purify the digests. RNA was quantified by absorbance at A_{260}.

Genes of interest for three calsarcin isoforms, *MYOZ1*, *MYOZ2*, and *MYOZ3*, were selected for analysis because they bind z-disc proteins such as calcineurin affecting muscle fiber type and contractile activity. Quantitative real time PCR (qRT-PCR) of gene expression for candidate genes was done by TaqMan® (Applied Biosystems, Foster City, CA) methods in an Applied Biosystems Step One Plus® instrument. Reactions, contained RNA-to-CT 1-Step® reagent, an adult skeletal muscle reference standard (commercially prepared skeletal muscle RNA; Ambion, Inc), and individual primer-probe sets for *MYOZ1*, *MYOZ2*, *MYOZ3*, and *HPRT1* (Thermo-Fisher-Applied Biosciences). *HPRT1* is known as a "housekeeping gene" utilized for normalization of data. The comparative CT (ΔΔCT) method was used to quantify the RNA expression of *MYOZ1, 2, 3* (see below). Their relative expression quantities were determined through the comparison of their concentration threshold (C_T) with the "housekeeping" gene, *HPRT 1*. First, standardized plots were prepared, comparing the slopes of *MYOZ1, 2, 3* with *HPRT 1* for amplification properties. Properties of amplification for each gene are shown in **Table 1**. The percent efficiencies for all assayed genes were greater than 90% and within 10% of one another, indicating that quantitative comparisons could be made by the ΔΔCT method (Livak, Schmittgen, 2001). All quantification assays were calibrated with the adult skeletal muscle reference standard. Standards were 1, 10, and 100 ng of human skeletal muscle RNA for comparison of amplification plots. **Figure 2.B.** represents the amplification plot for *MYOZ1*, **Figure 2.C.** is the plot for *MYOZ2*, and **Figure 2.D.** displays the amplification plot of *MYOZ3*. **Figure 3** compares the standard curves for all 3 genes of interest and the internal control, verifying that amplification efficiencies are within 10%.

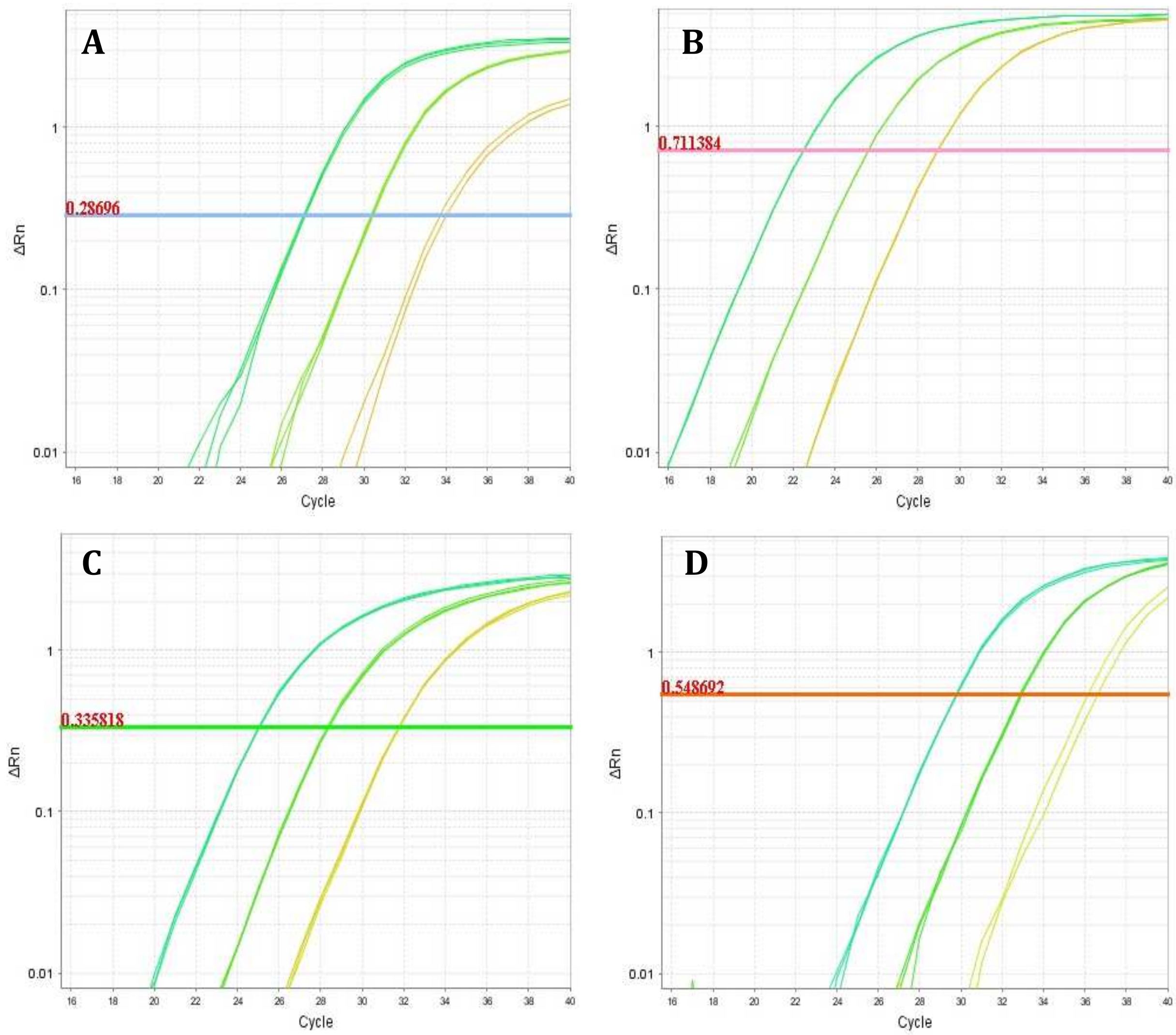


Figure 2. Standard Amplification Plots for Calibration of Calsarcin Gene Expression. Standard human skeletal muscle RNA amounts were 1ng, 10ng, and 100ng. Amplification thresholds, represented by the horizontal lines, are the level of fluorescence set low enough to be within the exponential growth region of the curve and above the baseline. **A.** Internal Control Gene HPRT1. **B.** *MYOZ1*. **C**. *MYOZ2*. **D.** *MYOZ3*.

Table 1 contains the summary of amplification properties for each gene. The slopes of standard curves were considered parallel and all efficiency percentages greater than 90% so that conditions for quantification by the ΔΔCT method were met and applicable for the quantification of expression of the three genes of interest.

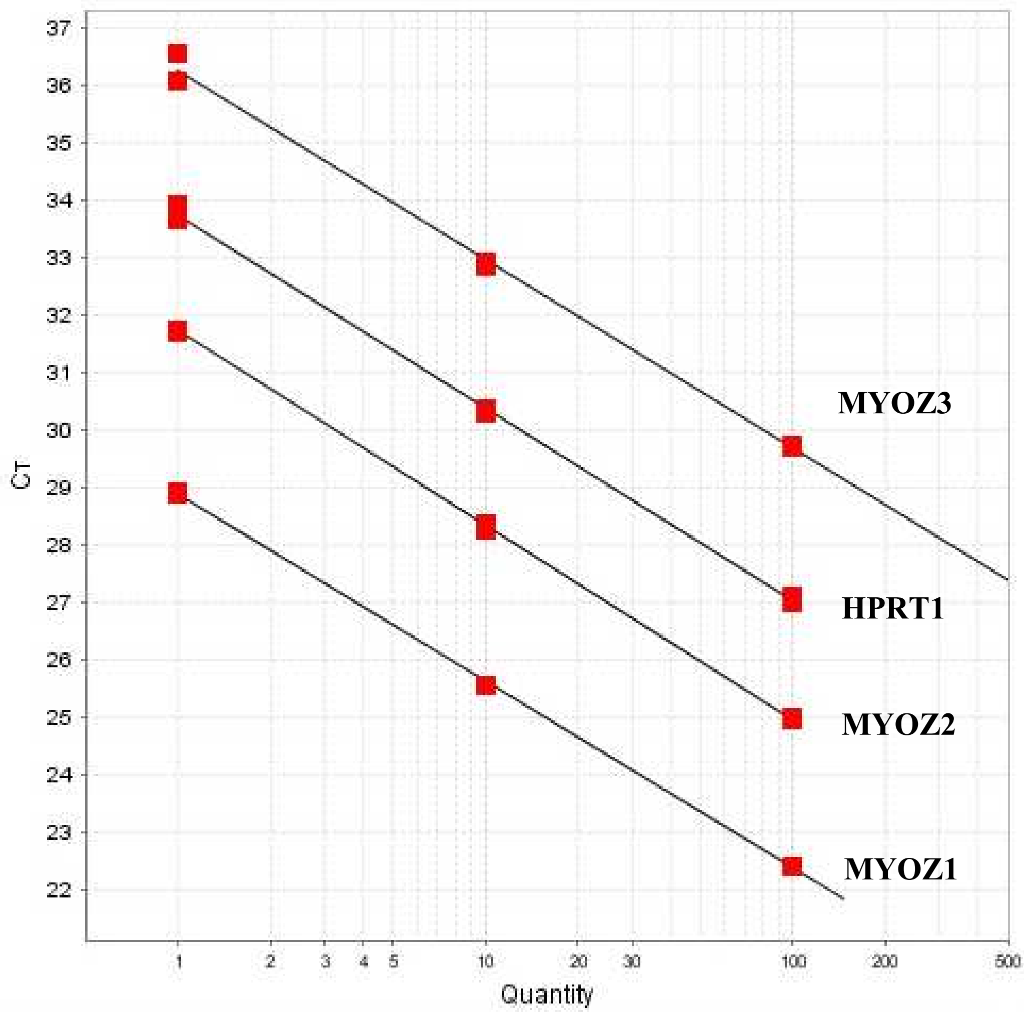


Figure 3. Standard Curves for HPRT1, MYO1, MYOZ2, MYOZ3. The thresholds cycles (C_T) for HPRT1, MYOZ1, MYOZ2, and MYOZ3 are plotted against their corresponding samples to illustrate slope parallelism.

Table 1. Comparison of Amplification Properties for HPRT1, MYOZ1, MYOZ2, and MYOZ3.

Gene	Slope	Y-Intercept	R^2	Efficiency %
HPRT1	-3.364	33.761	0.999	98.262
MYOZ1	-3.254	28.887	1.00	102.919
MYOZ2	-3.374	31.733	1.00	97.884
MYOZ3	-3.287	36.258	1.00	101.461

The following equations were used to calculate the fold changes in RNA expression:

Fold Change = $2^{-\Delta\Delta C_T}$ in which:

ΔC_T (Masseter Muscle Sample) = C_T (*MYOZ1*, Masseter RNA) – C_T (*HPRT1*, Masseter RNA)

ΔC_T (Masseter Muscle Sample) = C_T (*MYOZ2*, Masseter RNA) – C_T (*HPRT1*, Masseter RNA)

ΔC_T (Masseter Muscle Sample) = C_T (*MYOZ3*, Masseter RNA) – C_T (*HPRT1*, Masseter RNA)

ΔC_T (Calibrator Skeletal Muscle Sample) = C_T (*MYOZ1*, Skeletal Muscle RNA) – C_T (*HPRT1*, Skeletal Muscle RNA)

ΔC_T (Calibrator Skeletal Muscle Sample) = C_T (*MYOZ2*, Skeletal Muscle RNA) C_T (*HPRT1*, Skeletal Muscle RNA)

ΔC_T (Calibrator Skeletal Muscle Sample) = C_T (*MYOZ3*, Skeletal Muscle RNA) – C_T (*HPRT1*, Skeletal Muscle RNA)

$\Delta\Delta C_T$ = ΔC_T (Masseter Muscle Sample) – ΔC_T (Commercial Skeletal Muscle Sample)

2-ΔΔCT = 2-[ΔCT (Masseter Muscle Sample)-ΔCT (Commercial Skeletal Muscle Sample)]

With the completion of qRT-PCR, relative quantities, from the fold change data, are presented for comparison of *MYOZ1, 2,* and *3* expressions between each subject.

4.5 Statistical Analysis

ANOVA and unpaired t-tests were used to determine the significance of expression between MYOZ genes and between the *ACTN3* R577X genotypes within subject groups. ANOVA tests were done between sagittal and vertical malocclusion classifications when comparing groups of three or greater. Unpaired t-tests were used for comparisons between two subject groups. Differences were considered significant when p-values were ≤ 0.05.

Kendall Tau analyses were used to determine correlations between fiber-type percent occupancy and *MYOZ* expression levels in subjects grouped by malocclusion classification and masseter muscle composition. Graph Pad™ software was used to calculate p-values for the correlation data.

CHAPTER 5

RESULTS

5.1 Results Overview

Masseter muscle samples of 41 orthognathic surgery patients were utilized in this study. Of these patients, 15 were male and 26 were female with an average age of 25.8 ± 11.1 years. There were a total of 28 Class II subjects, which included 10 with normal bites, 12 open bites, and 6 deep bites, and 13 Class III subjects comprised of 3 with normal bite malocclusion, 4 open bite and 6 deep bite malocclusion. The genotype frequencies of R577X within the study population were 29% CC (RR), 42% TC (RX), and 29% TT (XX).

Genes of interest for three calsarcin isoforms, *MYOZ*1, *MYOZ*2, and *MYOZ*3, were selected for analysis because they bind Z-disc proteins such as calcineurin affecting muscle fiber type and contractile activity. The main aim of this project was to determine whether expression of the three calsarcin genes differs between subjects with RR, RX and XX genotypes for the *ACTN3* gene R577X polymorphism, as well as between sagittal and vertical classes of malocclusion, facial asymmetry and TMD. RT-PCR for the three genes was performed on the masseter muscle samples described above. Comparisons between subjects indicated that calsarcin gene expression only showed significance between Class II and Class III samples for the *MYOZ2* gene. Significant differences were not detected between all other vertical or sagittal classes of malocclusion, as well as facial asymmetry or with TMD-related myalgia. Only among subjects with the RX genotype did expression differ significantly between males and females by an unpaired *t*-test.

5.2 *MYOZ* mRNA Expression

The relative amount of mRNA, quantified by TaqMan RT-PCR, in both right- and left-sided masseter muscle samples was determined for *MYOZ*1, 2, and 3. The average range of expression values for *MYOZ*1, 2, and 3 were 0.97 to 8.61, 0.19 to 3.81, and 15.38 to 120.53, respectively. Expression relative to a quantitative standard was highest for *MYOZ3* (**Figure 4**) among the calsarcin isoforms (*MYOZ1*, 3.58 ± 1.45; *MYOZ2*, 1.20 ± 0.76; *MYOZ3*, 46.02 ± 23.18). Notably, *MYOZ3* was expressed at substantially greater relative levels in masseter muscle than in skeletal limb muscle, which had been used as a standard.

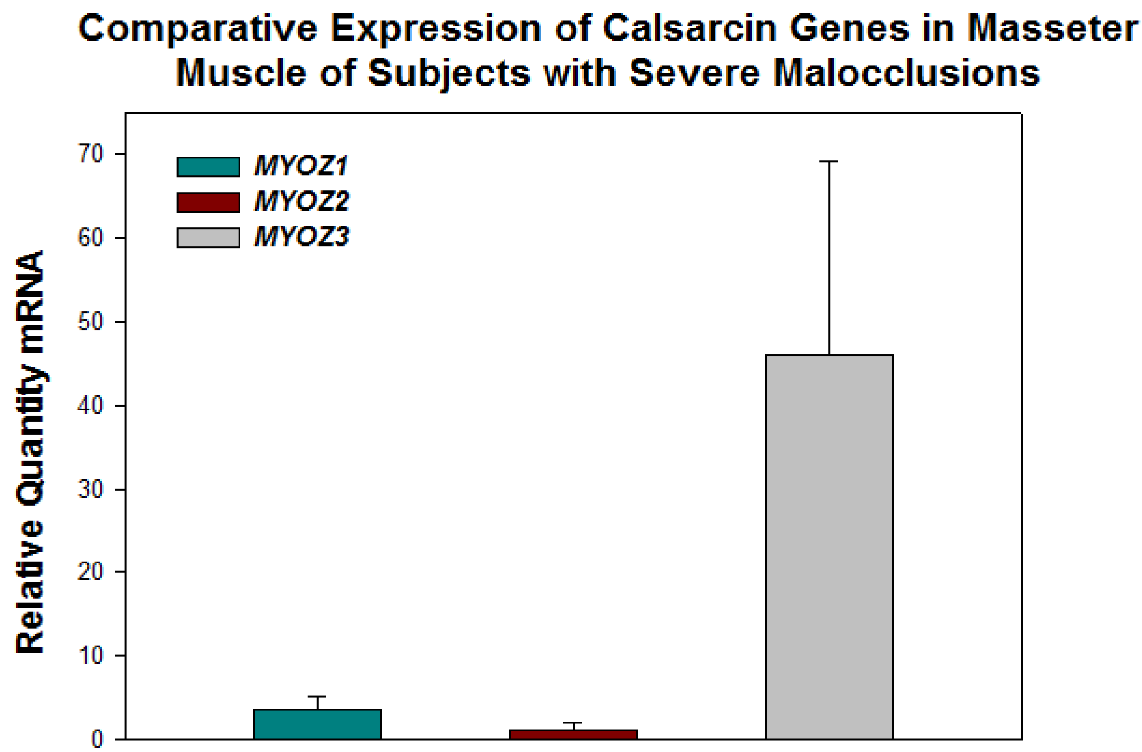


Figure 4. Differential relative gene expression between *MYOZ* isoforms.

5.3 Comparative Expression of *MYOZ* Genes by *ACTN3* 577XGenotype

Expression of calsarcin genes was further characterized by the *ACTN3* 577X genotypes; see **Table 2** and **Figure 5**. ANOVA analyses, conducted between all calsarcin genes by genotype combinations, revealed no significant differences in expression. These tests were followed by unpaired *t*-tests, which also illustrated no significant differences in any *MYOZ* expression between sets of subjects with different genotypes.

Table 2. Mean Calsarcin Expression Levels by R577X Genotypes			
	*MYOZ*1	*MYOZ*2	*MYOZ*3
RR (n=12)	3.84 ± 1.93	1.31 ± 0.71	51.82 ± 31.00
RX (n=17)	3.52 ± 1.39	1.04 ± 0.83	43.84 ± 20.37
XX (n=12)	3.42 ± 0.78	1.33 ± 0.66	43.3 ± 15.48

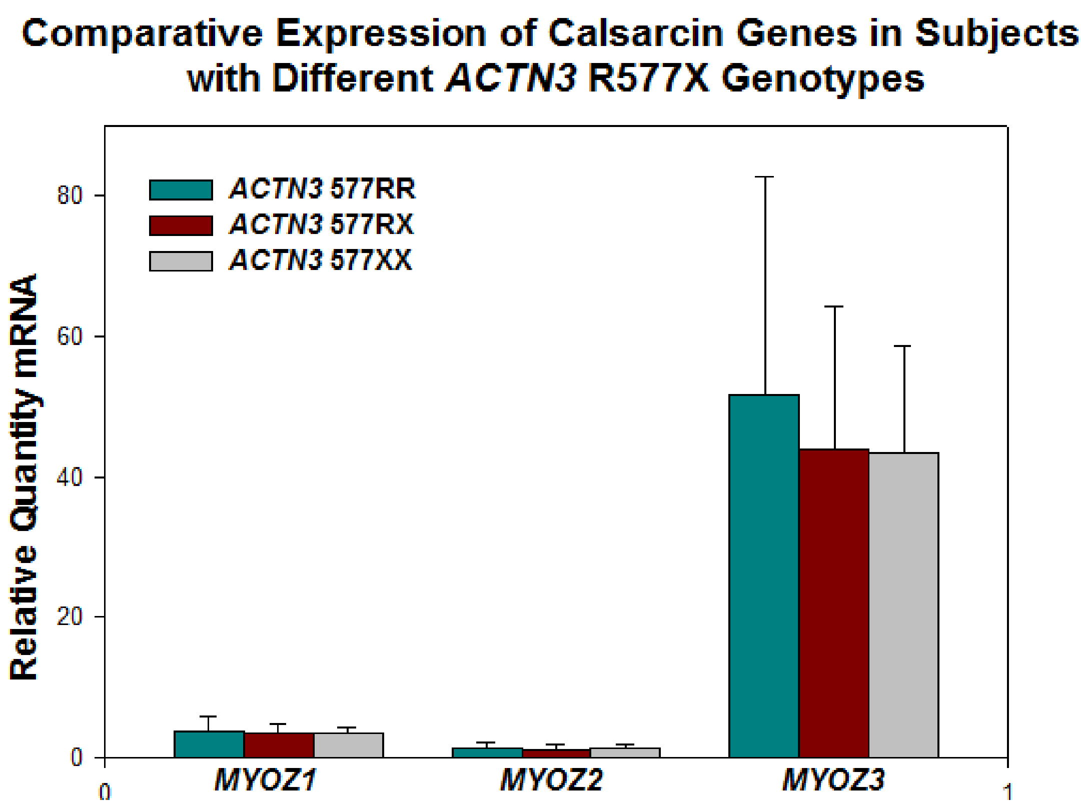


Figure 5. Graph depicting the differential relative expression between genotypes and *MYOZ* isoforms

5.4 Comparative Expression of *MYOZ* Genes by Genotype and Sex

Expression of *MYOZ*1, 2, and 3 were then organized by genotype and sex. Significant calsarcin expression differences were not detected between *ACTN3* R577X genotypes and sex using ANOVA analyses. However, differences were found for *MYOZ3* expression where relative quantities in males, but not females, decreased progressively from the *ACTN3* RR, to RX, and XX genotypes, **Figure 6**. Among subjects with the RX genotype, expression differed significantly between males and females by an unpaired *t*-test (**Figure 7**, p=0.01). Comparison of values between RR and XX males had p<0.2, but was under powered (0.27). Power analysis at 0.8 indicates that n=16 for each group would be needed to attain significance (p=0.05).

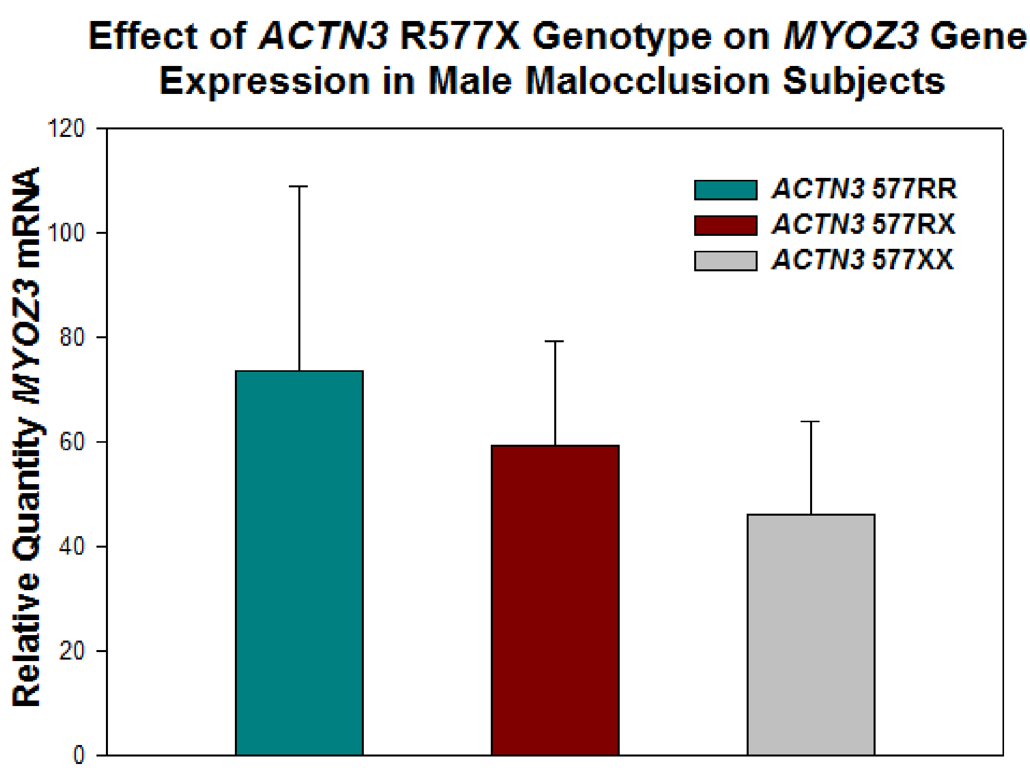


Figure 6. Graph depicting the progressive decrease in relative expression between genotypes and *MYOZ*3 isoform.

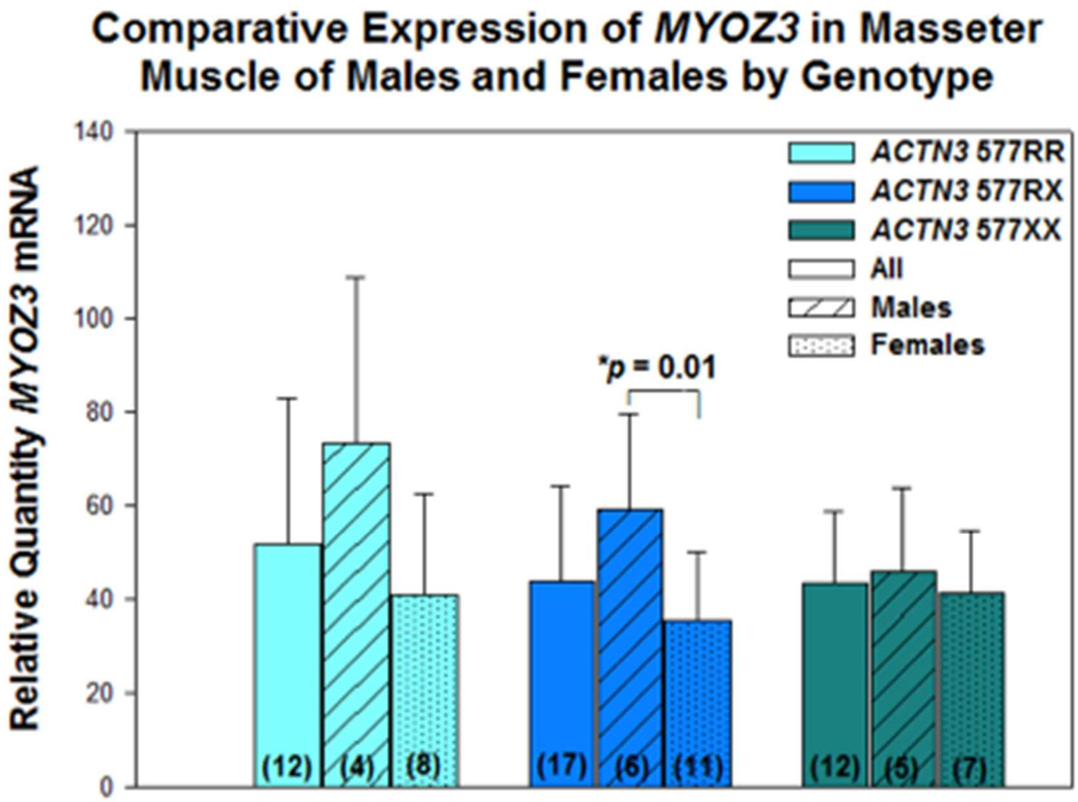


Figure 7. Graph depicting the differential relative expression between genotypes, sex, and *MYOZ* isoforms. Note the significant difference in expression between males and females for *MYOZ*2.

5.5 Comparative Expression of *MYOZ* Genes by Malocclusion

Comparisons between calsarcin gene expression and vertical or sagittal classes of malocclusion, with facial asymmetry or with TMD-related myalgia were made by unpaired *t*-tests and ANOVA analyses. No significant differences were detected between *MYOZ* gene expression and vertical malocclusions, facial asymmetry and TMD-related myalgia. For comparisons between sagittal classes and calsarcin expression, a statistically significant difference was detected between *MYOZ*2 and Class II, Class III malocclusions (p=0.05). Sagittal differences were compared further by ANOVA analyses of Class II male, Class II female, Class III male, and Class III female for each calsarcin gene. A statistically significant difference was only detected for *MYOZ*3 with a probability of 0.02 for Class III between males and females.

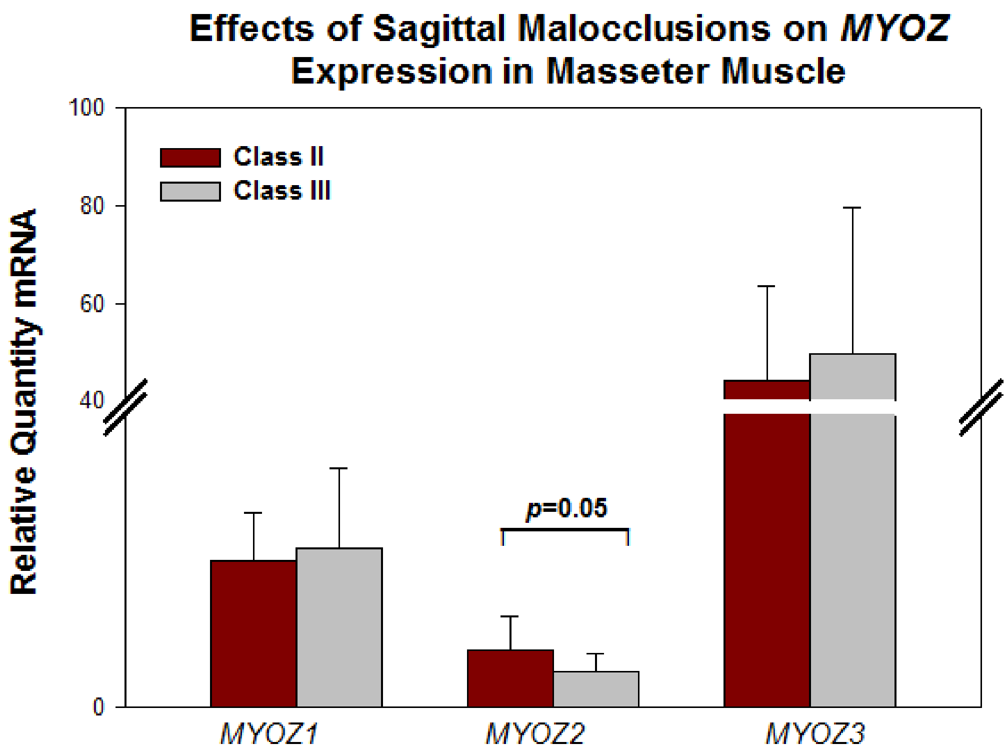
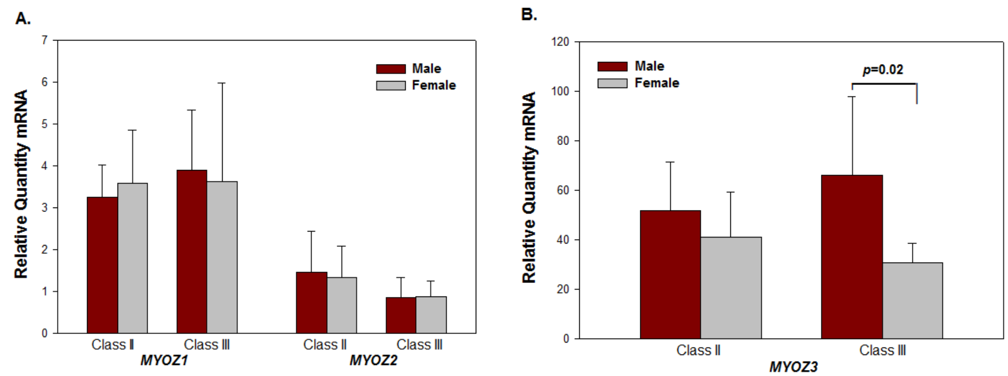


Figure 8. Graph depicting the differential relative expression between genotypes, sagittal malocclusions, and *MYOZ* isoforms.

Effects of Sagittal Malocclusion and Sex on *MYOZ* Expression in Masseter Muscle



Figures 9A. and 9B. Graphs depicting the differential relative expression between sagittal malocclusions, sex, and *MYOZ* isoforms. Note the significant difference in expression between Class III males and females for *MYOZ*3.

5.6 Correlation Analyses for Fiber-Type and *MYOZ* Gene Expression

The final aim of this study was to determine correlation values between calsarcin gene expression and fiber-type properties in masseter muscles of malocclusion subjects. Correlation analyses comparing fiber type mean % occupancy with calsarcin gene expression revealed a significant positive relationship between *MYOZ2* and type I (slow-twitch) fibers (**Figure 10**). Correspondingly, a significant correlation of *MYOZ2* expression with type IIA and IIX (fast-twitch) fibers was negative (**Figure 11**).

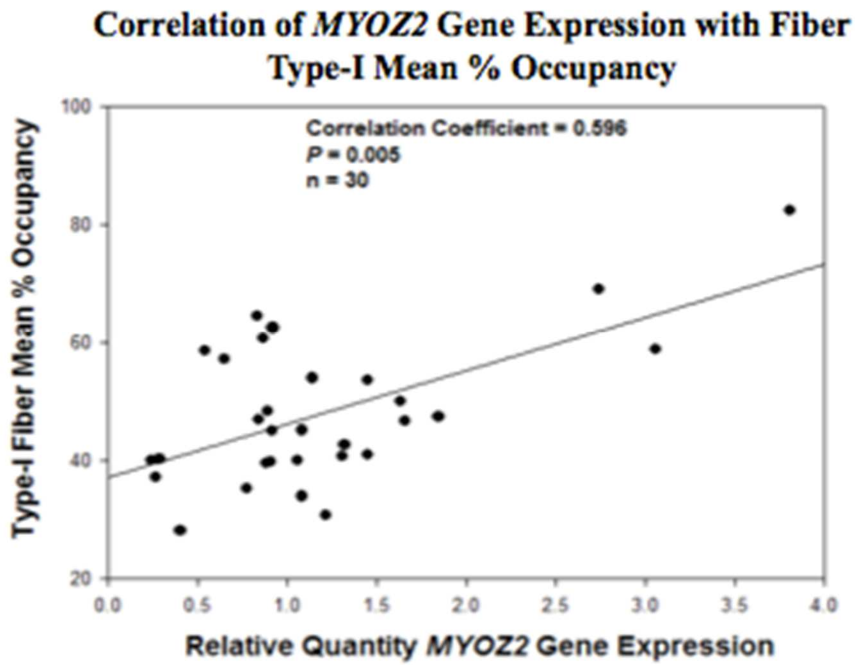


Figure 10. Graph depicting the positive correlation between *MYOZ*2 expression and Fiber-Type I Mean % Occupancy.

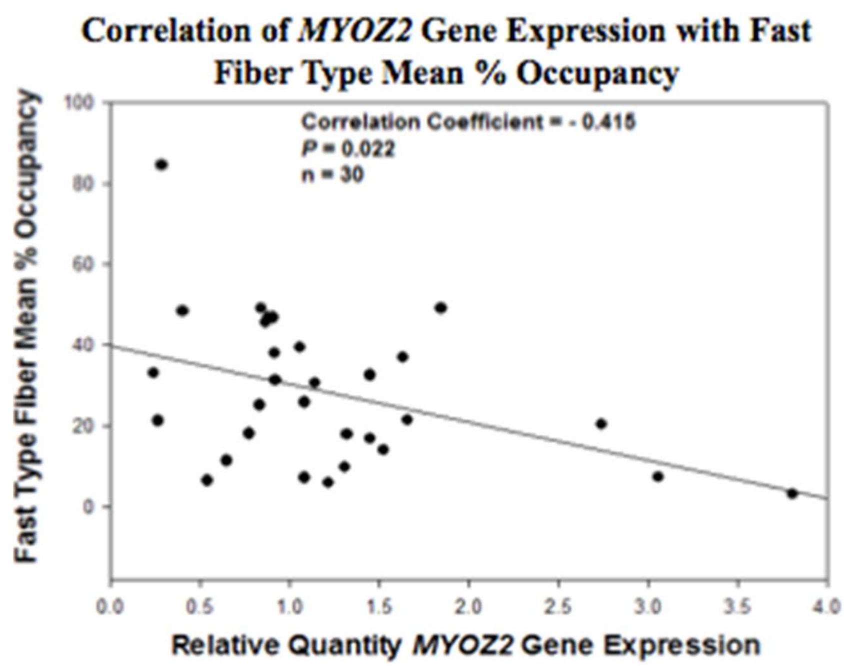


Figure 11. Graph depicting the negative correlation between *MYOZ*2 expression and Fast Fiber-Type Mean % Occupancy.

CHAPTER 6

DISCUSSION

A single nucleotide polymorphism (SNP) produces the R577XX polymorphism in *ACTN*3 to produce a stop codon and loss of its protein product, yet subjects carrying the mutation are seemingly unaffected. Individuals possessing this mutation display heightened endurance, which was replicated in *ACTN*3 knockout mice. Accordingly, the R577X polymorphism, with its known effects on fiber type proportions and muscle performance, has provided an effective way to study the influence of *ACTN3* and associated Z-disc protein genes on abnormal craniofacial morphology.

Using the knockout mouse model, the molecular bases for the effects of this nonsense mutation were determined. With loss of *ACTN*3, the *ACTN*2 is upregulated. Since it was demonstrated that calsarcins preferentially bind *ACTN2,* there is a surge in free calcineurin that leads to the phenotypic shift of fast muscle fibers toward the slow myogenic program (Seto et al., 2013). In understanding the integral role calsarcins play in this interaction, we sought to analyze the expression of three calsarcin genes (*MYOZ1*, *2*, and *3*) in masseter muscle of orthognathic surgery patients with each *ACTN3* SNP genotype.

Expression levels for each calsarcin gene were determined for 41 subjects and then further subdivided by genotype and sex. The overall expression of the *MYOZ* isoforms revealed that *MYOZ*3 expression, relative to a skeletal limb muscle standard, was significantly greater in masseter muscle. High amounts of *MYOZ3* in masseter, relative to a limb muscle standard, suggest that calsarcin-3 may be important in masticatory muscle structure and function.

Calsarcin gene expression was then characterized by genotype, exposing no significant expressional differences between subjects with each *ACTN*3 R577X genotype. The results of this comparison are consistent with *ACTN*3 knockout mice that, while demonstrating an upregulation of α-actinin-2, showed no difference in expression of *MYOZ*1 when compared to *ACTN*3 R577RR control mice (Seto et al., 2013). Therefore, the mutational effects of the 577XX polymorphism in *ACTN*3 may act in human masseter muscle as in limb muscle of knockout mice.

While no significant calsarcin expression differences were detected when data was compared by genotype and sex, an interesting trend became apparent.*MYOZ*3 expression in males decreased progressively from the *ACTN3* RR, to RX, and XX genotypes. This trend was not replicated in female subjects, or when analyzing the overall population by genotype, as described above. While this data may suggest that the *ACTN3* R577X polymorphism may affect *MYOZ3* expression in males of the malocclusion patient population, an increased sample of male subjects would be needed to determine if this trend has true significance. Among subjects with the RX genotype, expression differed significantly between males and females, but this was not observed for the RR and XX genotype. These results are not definitive in that the power analysis indicates an additional 16 patients would be needed to attain significance for all genotypes.

Final comparisons were made for calsarcin gene expression and vertical or sagittal classes of malocclusion, with facial asymmetry or with TMD-related myalgia. No significant differences were detected between *MYOZ* gene expression and vertical malocclusions, facial asymmetry and TMD-related myalgia. However, for comparisons between sagittal classes and calsarcin expression, a statistically significant difference was detected between *MYOZ*2 and Class II, Class III malocclusions (p=0.0498). Sagittal differences were compared further by ANOVA analyses of Class II male, Class II female, Class III male, and Class III female for each calsarcin gene. A statistically significant difference was only detected for *MYOZ*3 between Class III males and females with a probability of 0.02. Further studies would be needed with greater subject numbers to determine if there is a true trend to these findings. This data would indicate that calsarcin composition of the Z-disc may not directly affect musculoskeletal phenotype in subjects with severe malocclusions

In completing the final aim of this project, correlation analyses comparing fiber type mean % occupancy and calsarcin gene expression were performed. *MYOZ2* expression showed a significant positive correlation with type I (slow-twitch) fiber occupancy. Correspondingly, a significant negative correlation of *MYOZ2* expression with type IIA and IIX (fast-twitch) fibers was revealed. The muscle-specific expression of each calsarcin may lend to the understanding of this result. *MYOZ2* is the only isoform found in both cardiac muscle and slow-twitch skeletal muscle, while *MYOZ1* and *MYOZ3* are both found in skeletal muscle with a predilection towards fast-twitch skeletal muscle (Frey et al., 2004).

To our knowledge, this is the first report on the high degree of expression of *MYOZ3* in masseter muscle. Results suggest that there are potential differences in skeletal muscle and masseter with regard to calsarcin distribution. Further studies would be needed to determine whether the calcineurin pathway in knockout mice is identical to human ACTN3-null humans.

CHAPTER 7

CONCLUSIONS

1. RNAs for the three calsarcin isoforms are differentially expressed in human masseter muscle, where greatest relative quantities were seen for *MYOZ3*. High amounts of *MYOZ3* in masseter, relative to a limb muscle standard, suggest that calsarcin-3 may be important in masticatory muscle structure and function.

2. Calsarcin gene expression in masseter muscle did not differ significantly between skeletal classes of malocclusion and facial asymmetry in our patient population. This data indicates that calsarcin composition of the Z-disc may not directly affect musculoskeletal phenotype in subjects with severe malocclusions.

3. Our data suggest that the *ACTN3* R577X polymorphism may affect *MYOZ3* expression in males of the malocclusion patient population.

4. Expression of *MYOZ2* (calsarcin-1) was strongly correlated with slow fiber-type occupancy in masseter muscle of our patient population. Correspondingly, a significant negative correlation of *MYOZ2* expression with type IIA and IIX (fast-twitch) fibers was revealed. The muscle-specific expression of each calsarcin may lend to the understanding of this result in that *MYOZ2* is the only isoform found in slow-twitch skeletal muscle (Frey et al., 2004).

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APPENDICES

APPENDIX A
Jaw Pain and Function Questionnaire

Jaw Pain and Function Questionnaire – English Version

Jaw symptom and oral habit questionnaire	
First name : L	Date :
Last name : VG	19/02/2013
Identification : 004	
Instructions: Please check the appropiate answer to the following questions	Examiner :
	R. Nicot

A	Jaw pain questions	Doesn't hurt at all	Hurts a little	Hurts a lot	Almost unbearable	Unbearable pain without relief
1	Does it hurt when you open wide or yawn ?	X				
2	Does it hurt when you chew or use the jaws ?	X				
3	Does it hurt when you are not chewing or using the jaws ?	X				
4	Is your pain worse on waking ?	X				
5	Do you have pain in front of the ears or earaches ?	X				
6	Do you have jaw muscle (cheek) pain?	X				
7	Do you have pain in the temples ?		X			
8	Do you have pain or soreness in the teeth ?	X				
B	**Jaw function questions**	**No**	**Maybe a little**	**Quite a lot**	**Almost all the time**	**All the time without stopping**
9	Do your jaw joints make noise so that it bothers you or others?	X				
10	Do you find it difficult to open your mouth wide ?	X				
11	Does your jaw ever lock closed so you cannot open it?		X			
12	Does your jaw ever lock open so you cannot close it?	X				
13	Do you have a problem with your bite being uncomfortable?	X				

APPENDIX B
Summary of Subject Diagnosis

Subject	Gender	Age	Sagittal	Vertical	Midline Asymmetry > 3mm	JPF Score	TMD Signs	Apnea	Race	Genotype
1	Male	17	Class II	Normal	Midline R	0	No	No	FR-C	CC
2	Female	17		Open	Minor	2	No	No	FR-C	TC
3	Female	41	Class II	Open	Minor	2	No	Yes	FR-C	TC
4	Female	53	Class III	Open	Minor	0	No	No	FR-C	TT
5	Female	18	Class III	Deep	Midline R	0	No	No	A	CC
6	Male	23	Class III	Deep	Minor	3	No	No	FR-C	TT
7	Male	45	Class II	Open	No	0	No	Yes	FR-C	TT
8	Male	29	Class III	Normal	Midline L	0	No	No	A	TC
9	Female	24	Class II	Deep	Midline L	0	No	No	FR-C	TC
10	Female	47	Class II	Deep	No	9	Yes	No	FR-C	TC
11	Male	17	Class II	Normal	No	5	Yes	No	FR-C	TT
12	Female	24	Class II	Open	Minor	5	Yes	No	FR-C	CC
13	Female	17	Class II	Open	No	4	No	No	FR-C	TC
14	Female	35	Class II	Open	No	5	Yes	No	FR-C	CC
15	Male	17	Class III	Normal	No	1	No	No	FR-C	TT
16	Female	14	Class III	Open	Minor	2	No	No	FR-C	TC
17	Female	34	Class II	Deep	No	4	No	No	FR-C	TC
18	Female	40	Class II	Open	No	3	No	No	FR-C	TT
19	Female	30	Class II	Open	Minor	3	No	No	FR-C	CC
20	Male	26	Class II	Open	Midline R	0	No	No	FR-C	TC
21	Female	45	Class II	Normal	No	1	No	No	FR-C	TC
22	Female	20	Class II	Open	No	2	No	No	A	N/A
23	Male	18	Class III	Open	Minor	1	No	No	FR-C	TC
24	Female	38	Class III	Open	Midline L	2	No	No	FR-C	TT
25	Female	15	Class II	Deep	Minor	0	No	No	FR-C	TC
26	Female	16	Class II	Normal	Minor	0	No	No	FR-C	TT
27	Male	36	Class II	Deep	Minor	3	No	No	FR-C	TC
28	Male	20	Class III	Open	Midline left	1	No	No	FR-C	CC
29	Male	15	Class II	Normal	Minor	0	No	No	FR-C	TC
30	Female	20	Class II	Open	Midline R	14	Yes	No	FR-C	TT
31	Female	15	Class II	Deep	No	1	No	No	Parsee	TT
32	Female	34	Class III	Deep	Minor	1	No	No	A	CC
33	Male	16	Class III	Normal	Midline-L	5	No	No	FR-C	TC
34	Female	18	Class II	Open	No	9	Yes	No	FR-C	TC
35	Female	15	Class II	Normal	Minor	0	No	No	FR-C	TT

36	Female	23	Class III	Deep	No	9	Yes	No	NA	N/A
37	Male	17	Class III	Open	Midline-R	4	No	No	FR-C	CC
38	Female	18	Class II	Normal	No	10	Yes	No	FR-C	CC
39	Male	20	Class II	Normal	Midline-R	0	No	No	FR-C A	CC
40	Male	21	Class II	Normal	Minor	0	No	No	FR-C NA	TT
41	Female	49	Class II	Normal	No	5	No	No	FR-C	CC

APPENDIX C
Expression of *MYOZ1* in Masseter Muscle Samples

	Relative Quantity of mRNA in Masseter Muscle				
Subject	Right Side	Left Side	Difference	Pooled Value	RL Average
1	0.92	4.32	3.39	—	2.62
2	4.73	3.93	0.80	—	4.33
3	0.98	1.14	0.16	—	1.06
4	2.01	2.10	0.09	—	2.05
5	2.32	1.70	0.63	—	2.01
6	4.34	4.22	0.12	—	4.28
7	3.02	2.66	0.36	—	2.84
8	4.18	4.49	0.31	—	4.33
9	2.85	2.34	0.51	—	2.59
10	2.90	2.98	0.08	—	2.94
11	3.88	3.53	0.35	—	3.70
12	5.63	5.24	0.39	—	5.44
13	1.40	2.40	1.00	—	1.90
14	2.96	1.28	1.69	—	2.12
15	3.46	2.70	0.76	—	3.08
16	1.26	3.49	2.22	—	2.38
17	—	—	Pooled	4.34	4.34
18	—	—	Pooled	3.44	3.44
19	—	—	Pooled	5.19	5.19
20	4.16	4.11	0.05	—	4.14
21	—	—	Pooled	3.73	3.73
22	—	—	Pooled	4.18	4.18
23	—	—	Pooled	0.97	0.97
24	4.10	4.51	0.41	—	4.30
25	—	—	Pooled	3.77	3.77
26	—	—	Pooled	3.52	3.52
27	—	—	Pooled	2.97	2.97
28	7.98	2.67	5.31	—	5.32
29	—	—	Pooled	4.45	4.45
30	2.46	4.82	2.36	—	3.64
31	3.67	4.11	0.44	—	3.89
32	2.75	2.14	0.61	—	2.44
33	5.54	No RNA	—	—	5.54
34	No RNA	6.15	—	—	6.15

35	4.43	4.34	0.09	—	4.39
36	13.56	3.66	9.89	—	8.61
37	4.59	3.05	1.54	—	3.82
38	0.95	2.25	1.30	—	1.60
39	3.39	3.28	0.10	—	3.33
40	1.78	2.12	0.34	—	1.95
41	3.24	3.80	0.57	—	3.52
Average	3.65	3.32	1.24	3.66	3.58
SD	2.40	1.18	1.99	1.07	1.45
N					41.00

APPENDIX D
Expression of *MYO2* in Masseter Muscle Samples

	Relative Quantity of mRNA in Masseter Muscle				
Subject	Right Side	Left Side	Difference	Pooled Value	RL Average
1	0.51	1.61	1.10	—	1.06
2	0.63	1.13	0.50	—	0.88
3	0.34	0.23	0.11	—	0.29
4	0.86	0.88	0.03	—	0.87
5	1.22	0.45	0.77	—	0.83
6	0.86	1.12	0.26	—	0.99
7	0.69	0.61	0.08	—	0.65
8	0.19	0.28	0.09	—	0.24
9	0.33	0.48	0.16	—	0.40
10	1.13	1.14	0.01	—	1.14
11	1.63	0.54	1.09	—	1.09
12	1.92	1.76	0.16	—	1.84
13	0.84	0.84	0.00	—	0.84
14	1.13	0.42	0.71	—	0.77
15	1.34	0.91	0.43		1.12
16	0.35	0.18	0.17	—	0.27
17	—	—	Pooled	1.61	1.61
18	—	—	Pooled	1.45	1.45
19	—	—	Pooled	2.74	2.74
20	1.07	1.57	0.50	—	1.32
21	—	—	Pooled	1.64	1.64
22	—	—	Pooled	0.76	0.76
23	—	—	Pooled	0.19	0.19
24	1.12	1.93	0.81	—	1.52
25	—	—	Pooled	1.31	1.31
26	—	—	Pooled	0.54	0.54
27	—	—	Pooled	3.81	3.81
28	2.69	0.62	2.07	—	1.66
29	—	—	Pooled	0.92	0.92
30	0.81	1.36	0.55	—	1.08
31	1.10	1.80	0.70	—	1.45
32	0.22	1.59	1.37	—	0.91
33	0.89	No RNA	—	—	0.89
34	No RNA	1.21	—	—	1.21

35	4.23	1.89	2.34	—	3.06
36	1.40	0.31	1.09	—	0.86
37	0.59	1.24	0.65	—	0.92
38	0.26	1.29	1.04	—	0.77
39	0.58	0.83	0.25	—	0.71
40	2.19	1.99	0.20	—	2.09
41	3.18	2.20	0.97	—	2.69
Average	1.14	1.08	0.63	1.50	1.20
SD	0.90	0.59	0.58	1.02	0.76
N					41.00

APPENDIX E
Expression of *MYOZ3* in Masseter Muscle Samples

	Relative Quantity of mRNA in Masseter Muscle				
Subject	Right Side	Left Side	Difference	Pooled Value	RL Average
1	19.74	41.96	22.22	_	30.85
2	32.84	37.85	5.02	_	35.35
3	18.43	12.32	6.11	_	15.38
4	22.70	21.73	0.97	_	22.22
5	35.15	15.78	19.37	_	25.46
6	77.64	82.27	4.63	—	79.95
7	42.14	29.87	12.27	_	36.00
8	68.59	65.25	3.34	_	66.92
9	23.65	19.34	4.31	_	21.49
10	21.86	20.33	1.52	_	21.09
11	67.24	27.03	40.21	_	47.13
12	55.46	57.44	1.98	_	56.45
13	27.58	26.60	0.97		27.09
14	24.30	10.01	14.28	_	17.15
15	33.95	24.60	9.36	_	29.28
16	21.21	28.56	7.35	_	24.89
17	_	_	Pooled	39.08	39.08
18	_	_	Pooled	40.00	40.00
19	_	_	Pooled	90.09	90.09
20	90.29	94.55	4.25	_	92.42
21	_	_	Pooled	52.44	52.44
22	_	_	Pooled	34.63	34.63
23	_	_	Pooled	33.53	33.53
24	43.64	49.13	5.48	_	46.38
25	_	_	Pooled	56.74	56.74
26	_	_	Pooled	53.32	53.32
27	_	_	Pooled	51.20	51.20
28	148.18	38.59	109.60	_	93.39
29	_	_	Pooled	72.05	72.05
30	43.19	71.22	_	_	28.03
31	50.38	75.74	25.36	_	63.06
32	23.67	37.31	13.63	_	30.49
33	39.67	no RNA	–	–	39.67
34	no RNA	61.25	_	_	61.25

35	36.80	37.19	0.39	_	36.99
36	47.32	21.15	26.17	_	34.24
37	92.95	148.10	55.15	_	120.53
38	21.55	63.47	41.91	_	42.51
39	49.32	48.42	0.89	_	48.87
40	37.25	37.18	0.07	_	37.22
41	34.26	29.42	4.84	_	31.84
Average	45.03	44.45	15.77	52.31	46.02
SD	27.73	28.82	22.78	16.82	23.18
N					41.00

APPENDIX F
Comparison of *MYOZ* Expression by Genotype

Correlation Analyses for *MYOZ* Expression Levels and Genotype				
	Genotype	***p*-Value**	**Statistical Analysis**	**Subject Number**
***MYOZ*1**	RR vs. XX	0.50	unpaired *t*-test	RR=12 RX= 17 XX=12
	RR vs. RX	0.61		
	RX vs. XX	0.84		
	RR vs. RX vs. XX	0.77	ANOVA	
***MYOZ*2**	RR vs. XX	0.97	unpaired *t*-test	
	RR vs. RX	0.36		
	RX vs. XX	0.33		
	RR vs. RX vs. XX	0.53	ANOVA	
MYOZ3	RR vs. XX	0.40	unpaired *t*-test	
	RR vs. RX	0.41		
	RX vs. XX	0.94		
	RR vs. RX vs. XX	0.61	ANOVA	

APPENDIX G
Comparison of *MYOZ* Expression by Genotype and Sex

Correlation Analyses for MYOZ Expression Levels by Genotype and Sex					
	Genotype	Sex	Subject Number	*p*-Value	Statistical Analysis
*MYO*1	RR	Male	4	0.97	ANOVA
		Female	8		
	RX	Male	6		
		Female	11		
	XX	Male	5		
		Female	7		
MYO2	RR	Male	4	0.78	ANOVA
		Female	8		
	RX	Male	6		
		Female	11		
	XX	Male	5		
		Female	7		
MYO3	RR	Male	4	0.056	ANOVA
		Female	8		
	RX	Male	6		
		Female	11		
	XX	Male	5		
		Female	7		

APPENDIX H
Comparison of *MYOZ* Expression by Sagittal Classification

Correlation Analyses for *MYOZ* Expression Levels and Sagittal Malocclusion				
	Sagittal Classification	**Subject Number**	***p*-value**	**Analysis**
***MYOZ*1**	Class II	28	0.55	unpaired *t*-test
	Class III	13		
MYOZ2	Class II	28	0.049*	unpaired *t*-test
	Class III	13		
***MYOZ*3**	Class II	28	0.48	unpaired *t*-test
	Class III	13		

APPENDIX I
Comparison of *MYOZ* Expression by Sagittal Classification and Sex

Correlation Analyses for MYOZ Expression Levels by Sagittal and Sex					
	Sagittal	**Sex**	**Subject Number**	***p*-Value**	**Statistical Analysis**
***MYO*1**	Class II	Male	8	0.87	ANOVA
		Female	20		
	Class III	Male	7		
		Female	6		
MYO2	Class II	Male	8	0.29	ANOVA
		Female	20		
	Class III	Male	7		
		Female	6		
MYO3	Class II	Male	8	0.02*	ANOVA
		Female	20		
	Class III	Male	7		
		Female	6		

APPENDIX J
Comparison of *MYOZ* Expression by Vertical Classification

Correlation Analyses for *MYOZ* Expression Levels and Vertical Malocclusion				
	Vertical Classification	***p*-Value**	**Statistical Analysis**	**Subject Number**
***MYOZ*1**	Deep vs. Open	0.67	unpaired *t*-test	Deep=10 Normal=13 Open=18
	Normal vs. Open	0.99		
	Normal vs. Deep	0.66		
	Deep vs. Normal vs. Open	0.89	ANOVA	
***MYOZ*2**	Deep vs. Open	0.37	unpaired *t*-test	
	Normal vs. Open	0.39		
	Normal vs. Deep	0.92		
	Deep vs. Normal vs. Open	0.62	ANOVA	
MYOZ3	Deep vs. Open	0.55	unpaired *t*-test	
	Normal vs. Open	0.71		
	Normal vs. Deep	0.65		
	Deep vs. Normal vs. Open	0.79	ANOVA	

APPENDIX K
Comparison of *MYOZ* Expression by Symmetry

Correlation Analyses for *MYOZ* Expression Levels and Symmetry				
	Symmetry	***p*-Value**	**Statistical Analysis**	**Subject Number**
***MYOZ*1**	+ Symmetry vs. - Symmetry	0.86	unpaired *t*-test	+ Symmetry=29 - Symmetry=12
***MYOZ*2**		0.18		
MYOZ3		0.21		

APPENDIX L
Comparison of *MYOZ* Expression by TMD

<table>
<tr><th colspan="5"><u>Correlation Analyses for MYOZ Expression Levels and Symmetry</u></th></tr>
<tr><th></th><th><u>Symmetry</u></th><th><u>p-Value</u></th><th><u>Statistical Analysis</u></th><th><u>Subject Number</u></th></tr>
<tr><td>MYOZ1</td><td rowspan="3">+ TMD vs.
- TMD</td><td>0.12</td><td rowspan="3">unpaired
t-test</td><td rowspan="3">+ TMD=8
- TMD=33</td></tr>
<tr><td>MYOZ2</td><td>0.66</td></tr>
<tr><td>MYOZ3</td><td>0.31</td></tr>
</table>