

EXPRESSION OF HISTONE DEACETYLASE 4 AND HISTONE
ACETYLTRANSFERASE 4 IN HUMAN MASSETER MUSCLE: RELATIONS TO
FIBER-TYPE COMPOSITION IN PATIENTS WITH MALOCCLUSIONS

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ABSTRACT

Significant advances have been made in orthodontics and oral maxillofacial surgery for the diagnosis and treatment of dentofacial deformities. However, compared with bone, the effect of muscles of the craniofacial complex in the development of dentofacial deformities has received little attention. Recently, cellular and molecular studies of the musculoskeletal interactions have been used to investigate the etiology of dentofacial malocclusions. In this study, we tested for relationships that might exist between gene expression of the chromatin modifying enzymes histone deacetylase-4 (HDAC4) and histone acetyltransferase-4 (MYST4) and expression of myosin heavy chain (MyHC) genes and fiber-type percent occupancy (%Occ) in masseter muscle of patients undergoing orthognathic surgery to correct severe dentofacial malocclusions. The diagnostic categories of malocclusion in sagittal and vertical dimensions were: 1) Deep bite-Class II (D2); 2) Deep bite-Class III (D3); 3) Normal bite-Class II (N2); 4) Normal bite-Class III (N3), 5) Open bite-Class II (O2); 6) open bite with Class III (O3). Relative quantities (RQs) of gene expression were determined by reverse transcriptase real time polymerase chain reaction (RT-PCR) in RNA extracts of masseter samples, previously analyzed by immunohistochemistry for %Occ values. By multivariate analysis, RQs of HDAC4 and MYST4 expression did not differ significantly between malocclusion types. However, multiple high positive and negative correlations were found for HDAC4 and MYST4 with MyHC expression and with fiber type %Occ. Significant correlations occurred for HDAC4 with: IIX and neonatal MyHCs respectively in N2 and N3 subjects; fiber types I, I/II and neonatal/atrial %Occ

respectively in D2 and N3, D2 and O3 subjects. Further investigations are needed to support evidence of these correlations and determine their significance toward diagnosis, treatment and relapse potential in the correction of dentofacial deformities.

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CHAPTER 1

INTRODUCTION

Dentofacial deformities in vertical dimension, such as open bite and deep bite, and sagittal dimension, such as Class I, II and III, are highly prevalent in patients seeking orthodontic treatment. The etiology of dentofacial deformities and malocclusion is multifactorial, and includes genetic, behavioral and environmental components. The soft tissue envelope, including the muscles of mastication, contributes to the development of dentofacial deformities by exerting variable pressures and forces related to physiological muscular activity such as mastication (Kiliaridis, 1995). An example of how facial muscle activity influences the osseous structure is the development of progressive open bite in children with Duchenne Muscular dystrophy (DMD) (Morel-Verdebout et al., 2007; Kiliaridis et al., 1998). The patients with DMD often develop progressive open bite due to decreasing muscle function as the condition exacerbates with age. Previous studies have demonstrated an association between particular masseter muscle fiber type, size and numbers and the development of open or deep bite malocclusion (Rowlerson et al., 2005; Suchak et al., 2009; Sciote et al., 2012). Human skeletal muscles are composed of mixtures of fiber types, and each fiber type is associated with specific functional characteristics. Epigenetic mechanisms are largely regulated by histone modification.

In eukaryotic cell nuclei, genomic DNA is folded in sequence around eight histone protein cores into nucleosomes. Nucleosomes are further compacted into a more packaged chromatin fiber. Histone modification through acetylation and/or deacetylation regulates transcription by changing the conformation of the chromatin. For example,

acetylation of histone allows transcriptional activation by making chromatin more accessible to transcription proteins. Improved knowledge of the epigenetic mechanism will aid the current understanding of the etiology of craniofacial deformities and malocclusion. Recent studies demonstrated that histone modifying enzymes may play a significant role during the differentiation of muscle fiber types (Pandorf et al., 2009). Characteristic variations in the composition of masseter muscle fiber types have been observed between open bite and deep bite malocclusions (Rowlerson et al., 2005; Sciote et al.; 2012, Suchak et al., 2009). Based on the current understanding, the functional properties of muscle fiber types may be a contributing factor in the development and maintenance of the craniofacial skeleton.

Various treatment modalities, including comprehensive orthodontic treatment, orthognathic surgery, distraction osteogenesis and functional appliance therapy, are used to correct dentofacial deformities. However, dental and skeletal relapse following orthodontic treatment alone or with orthognathic surgery is a common occurrence indicating the influence of soft tissue envelope such as craniofacial musculature (Bondemarka et al., 2007; Smithpeter et al. 2010; Zaher et al., 1994). Genetic screening of known muscular genes for particular dentofacial deformities may aid in diagnosis and treatment to predict the prognosis of various treatment modalities as the long-term success and stability rely heavily on muscular adaptation. If certain muscle genes are constantly expressed at a high level or low level with certain facial form, the information can be used to assess the muscle adaptability and the probability for relapse following treatment. Then, the patient is better informed to consent to a particular treatment or is spared the morbidity of surgery if it is highly likely that the correction will relapse. This

is the first study, to our knowledge, to investigate the relationship between chromatin modifying enzymes and fiber-type contractile proteins in human masseter muscle of various occlusal types. Studying the epigenetic phenomenon in fiber type differentiation and craniofacial development may enhance diagnosis, treatment planning and assessment of relapse potential in the correction of dentofacial malocclusion.

CHAPTER 2

LITERATURE REVIEW

2.1 Genetic and Epigenetic Basis of Skeletal development

Throughout its history, the foundation of orthodontic treatment was established on understanding the etiology of malocclusion and biological mechanisms of the craniofacial development and growth. Earlier studies by Hunter, and Enlow laid down a foundation for theories of craniofacial growth. Based on studies using Hunter's vital staining technique, Brash formulated Bone Remodeling Theory (Brash, 1934). Bone Remodeling Theory states that apposition at the surface is the major mechanism of skeletal growth. According to Brash, the growth of mandible and maxilla is characterized by deposition of bone at the posterior surfaces while calvarial growth is characterized by periosteal bony deposition and endosteal resorption of bone on the inner surface of the cranium. Brash rejected the role of suture and cartilage in the growth of the craniofacial skeleton. However, later studies acknowledged that Brash may have drawn erroneous conclusion by inappropriate experimental design with respect to the choice and age of the animal model (Mednick, 1956; Weinmann, 1947). In the 1940s, Weinmann and Sicher proposed the Sutural Theory that states the connective tissue and cartilaginous joints of the craniofacial skeleton, such as epiphyses and sutures, are the principal locations where primary growth of bone takes place (Weinmann et al., 1947; Sicher, 1960). Weinmann and Sicher proposed that sutures, cartilages and periosteum have intrinsic genetic control over craniofacial growth. Sicher considered the mandible to a bent long bone with mandibular condylar cartilage serving as the epiphyseal plate. However, several studies

of cranial sutures in an appropriate animal model demonstrated that the sutures were major sites but not the deterministic effector in the craniofacial skeletal growth (Moss 1954, 1957). As an alternative theory to the Sutural theory, Scott proposed the Nasal Septum Theory which states that the nasal septum is the most active growth site for craniofacial growth during early development (Scott, 1954). He stated that sutures are permissive, secondary, and compensatory sites of bone formation and growth, and suggested that the cartilage of the mandibular condyles behaves similarly to cranial base and nasal septal cartilages to directly determine the growth of the mandible. Until the 1950s, the experimental studies on craniofacial growth were largely histological and/or surgical making it impossible to understand the extent and location of genetic influence. However, the breakthroughs in genetics during the 1950s, such as the discovery of double helical structure of DNA by Watson and Crick and operon theory by Jacob and Monod, led to a “paradigm shift” in craniofacial research. Innovations in genetics, molecular biology and bioengineering allowed better understanding of the genetic control and regulatory pathways in craniofacial skeletal growth (Hodgkin, 1994; Roelofsen et al., 1995; Sachs, 1988).

Based on studies of bone and muscle physiology, Moss presented the Functional Matrix Hypothesis (FMH) in attempt to describe the controlling mechanism behind craniofacial growth (Moss, 1997A-D). Moss emphasized the importance of epigenetic interaction of intrinsic and extrinsic factors that results in variation in craniofacial form. According to FMH, the functional matrix is comprised of the tissues and cavities that carry out the function. The functional matrix is made up of capsular and periosteal matrixes. According to Moss, growth and maintenance of all skeletal tissues are always

secondary, compensatory and functionally obligatory to periosteal and capsular matrices. Moss stated parts of the functional matrix can be shown to have direct influence on the bone through the periosteal matrix by muscle function at the muscle insertion. He stated that periosteal functional matrix stimulates a skeletal unit through cellular mechanotransduction and electrochemical interaction. A static or dynamic loading triggers intercellular signal transmission and stimulates osteoblasts and osteoclast activity. According to computational bone biology, hard tissue adaptations can be explained through skeletal muscle contraction that activates mechanotransduction to elicit osseous changes by means of genetic, cellular and molecular interactions. According to Moss, the initial intracellular mechanotransduction through gap junctions transmits functional matrix information. Then, gap junctions of bone cell act as a cellular network to the multicellular bone adaptation processes. FMH may thus account for how epigenetic events are promoted by muscle contraction downward to the regulation of the bone cell genome. The coordination between intracellular signaling pathways and gene transcription results in cellular growth and differentiation. Muscle function depends to a large extent on the expression of genes required for tissue homeostasis and adaptation to patterns of contractile activity. Recently, studies of histone modifying proteins have shed light on the biochemical regulatory mechanisms for muscle fiber differentiation, musculoskeletal development and adaptation.

In human limb muscle, muscular strength and power have been shown to be significantly correlated to fiber type proportion (Gerdle et al., 1988). Recent studies of genetics and molecular biology have allowed a better understanding of the relationship between the muscular function and the development and maintenance of the skeletal

system. In a mice-model, significant correlations were noted between masseter muscle weight, mandibular body length, cranial vault length, and the mandibular shape index (Vecchione et al., 2007; 2010). Specifically, mice with a hyper-muscular phenotype due to loss of myostatin, a growth factor which negatively regulates muscle fiber size, were reported to have more brachycephalic and smaller cranial vaults, decreased maxillary length and a distinctive mandibular shape, significantly affecting the size and morphology of craniofacial growth. Variation in the contractile force and tonicity of muscle is influenced largely by the composition of muscle fiber types. Therefore, it is postulated that the distinct male facial morphology with anteriorly inclined mandibles and shorter anterior facial heights may be related to the greater bite force generated by greater diameter and cross sectional area of type II fibers in male (Tuxen et al., 1999). However, masticatory load doesn't seem to play as important role as muscle tone for postural purpose in humans as dentition is only in occlusion approximately 6 to 7 minutes in a day (Miyamoto et al., 1996).

2.2 Classification of Muscle Fiber Types

Muscle fibers are the primary cellular and contractile units of skeletal muscle. Muscle fiber types are classified based on histochemistry, immunohistochemistry and metabolic profiles. They are primarily dependent on the gene expression and protein composition of myosin heavy chains (MyHC), which are the motor molecules for contractility. A number of different genes encode various MyHC isoforms that differ in their functional properties, and are expressed in a tissue-specific and developmentally regulated manner. Isoform transitions are influenced by various factors, including

hormone levels, exercise, and physical damage. The typical classification system for skeletal muscle fiber types is based on the differential myofibrillar ATPase activity within the molecular head region of MyHCs. Different histochemical staining properties of the ATPase moieties fall into three main groups: type I, IIA and IIB. Type I slow twitch fibers produce low force but possess high fatigue resistance, which is suitable for postural function. Type IIA and IIB fast twitch fibers generate high but fatigable force for phasic activity used in rapid locomotion. The masseter belongs to the group of cranial masticatory muscles and differs from other skeletal muscle in fiber type composition. Sciote et al. (1994) classified human masticatory muscles into four fiber types including type I and type II. The type II fibers are comprised of type IIA and IIX, which is a fast isoform distinct from histochemical IIB. Type I/II hybrids express both type I and II MyHC proteins, and type neonatal/atrial (N/A) expresses a developmental and the cardiac alpha myosin (MyHC- α) isoforms. Rowlerson et al., (2005) using immunostaining techniques, demonstrated a reduction in the contribution of type II fiber phenotypes of masseter muscle in subjects with anterior open bites. Suchak et al. (2009) studied the gene expression for myosin heavy chains associated to each muscle fiber type and observed a reduction in type II fibers in subjects with anterior open bites and high mandibular plane angle. Recently, Raoul et al. (2011) reported significantly different fiber types between the left and right side masseter muscles of subjects with facial asymmetry, indicating the impact of muscle fiber composition on skeletal development. In addition to the MyHC proteins, a number of other proteins associate with the contractile apparatus of skeletal muscle fibers to determine slow and fast twitch properties. A global analysis of the human genome using an oligonucleotide microarray

indicates that expression of MyHC IIA and IIX along with several other genes related to a fast fiber-type composition are expressed at levels several fold greater in masseter muscle from subjects with deep bite over open bite malocclusion (Horton et al., unpublished results). Concomitantly, expression of MyHC- β , also known as MyHC-I, which is the major myosin of slow twitch fibers, along with several genes associated with slow fiber function is down regulated in masseter muscle from the deep bite subject. Signaling pathways that control fiber type-specific gene expression include calcineurin-dependent nuclear factor of activated T-cell (NFAT) transcription factors, *cis*-acting bidirectional promoters of MyHC gene transcription and muscle-specific microRNAs (miRNAs) found within the sequences of the MyHC genes themselves (Schiaffino et al., 2011).

2.3. Histone modification

In eukaryotic cell nuclei, genomic DNA is packaged into chromatin in the nucleus. DNA strands are wrapped around a histone core in a left handed superhelix and forms structural units called nucleosomes as shown in **Figure 1**. Nucleosomes are connected by a short piece of spacer DNA which allows the appearance of a ‘beads on a string’ under the electron microscope. The interaction between histone and nucleosome condenses DNA further into the chromatin fiber.

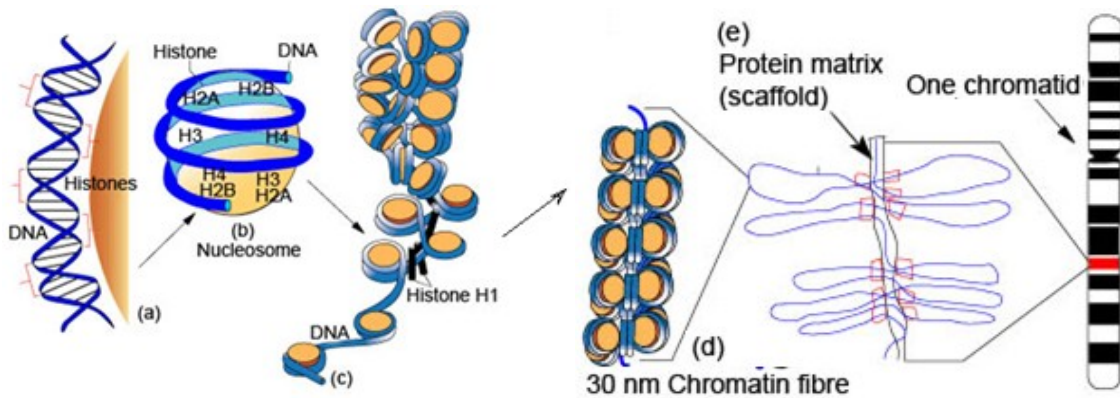
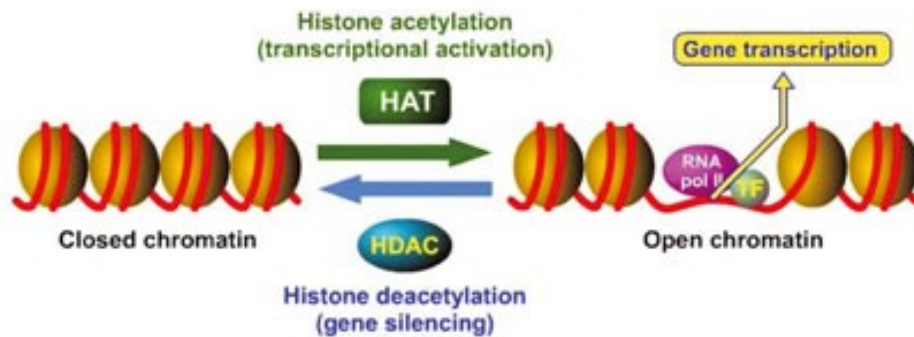


Figure 1. Molecular organization of eukaryotic chromosome

The formation of chromatin allows DNA to be compacted. Each human cell has approximately 1.8 meters of DNA, but the DNA is condensed into 90 micrometer of chromatin (Redon, 2002). DNA in chromatin of metaphase chromosomes is tightly folded during cell division as transcriptional activators or other transcription factors are unable to bind to their promotor elements. Histones undergo posttranslational modifications that alter their interaction with DNA and nuclear proteins resulting in transcriptional activation or repression. Modifications of histones include methylation, acetylation, phosphorylation, ubiquitination, SUMOylation, citrullination, and ADP-ribosylation. Hyperacetylated chromatin is transcriptionally active, and hypoacetylated chromatin is silent. Histone acetyltransferases (HATs) catalyze acetylation in a reversible manner to allow transcriptional activation as shown on **Figure 2**. Histone tails are normally positively charged due to amine groups present on their lysine and arginine amino acids. Addition of an acetyl group neutralizes the positive charge of lysine and reduces electrostatic attraction between the histone and the negatively charged DNA backbone, loosening the chromatin structure. Highly acetylated histones form more

accessible chromatin and tend to be associated with active transcription. Histone deacetylase (HDAC) removes acetyl groups from lysine residues on the amino-terminal part of the nucleosomal core histones (H2A, H2B, H3 and H4); this results in transcriptional repression by encouraging high-affinity binding between the histones and DNA backbone. Thus, the opposing activities of HDAC and HAT proteins create dynamic states of acetylation and deacetylation that alter chromosome structure and affect access of transcription factors to DNA.



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Figure 2. Transcriptional activation by HAT and gene silencing by HDAC

Although the regulation of gene transcription by modifying histones and chromatin structure appears to be the predominant function of HAT and HDAC, HAT and HDAC also interact with a variety of non-histone proteins to catalyze proteins that are not related to epigenetic regulation.

2.4. The role of histone modification during muscle differentiation

Myocyte enhancer factor 2 (MEF2) regulates transcription during muscle differentiation and activity-dependent remodeling of muscle fiber types (Potthoff et al., 2007). Class II HDAC-proteins repress MEF-2 transcriptional activation within the nuclei of skeletal muscle (Kao et al., 2001). Class I HDACs (1, 2 and 3) are expressed ubiquitously while Class II HDACs (4, 5, 7 and 9) have more restricted expression patterns and mediate interactions with other transcriptional cofactors. This pathway is further regulated by increased levels of intranuclear Ca^{+2} that activate a calmodulin dependent protein kinase, which then phosphorylates HDAC and promotes its efflux from nuclei to decrease the suppression of MEF2 activation of gene expression (Kao et al., 2001), particularly in slow-type fibers (Liu et al., 2005B). In summary, Class II HDACs provide a calcium-sensitive switch to control sets of genes regulated by MEF2. Other evidence indicates that the slow-twitch fiber formation is also enhanced by degradation of HDACs that is accompanied by the activation of MEF2 to activate the slow muscle fiber differentiation (Potthoff et al., 2007). In a study of individual HDAC variants, Liu et al. (2005A) showed that repetitive electrical stimulation of slow, but not fast, fiber types promotes translocation of green fluorescent protein (GFP)-labeled HDAC4, but not HDAC5 from the nucleus to the cytoplasm in cultured adult skeletal muscle fibers suggesting a preferential role for HDAC4 in the signaling pathway for regulation of slow fiber type gene expression. In support of this possibility, Cohen et al. (2009) have identified a group of muscle contractile and structural genes that are subject to HDAC4-dependent repression in both cultured myotubes and skeletal muscle. Whereas the latter mechanisms emphasize direct interaction between HDACs and MEF2 factors and not

DNA binding, Pandorf et al. (2009) have proposed a model for control of MyHC expression and fiber-type switching by epigenetic regulation of the acetylation and methylation of histones. In this work, H3 in regions at MyHC genes were isolated by chromatin immunoprecipitation. A schematic model for the control of slow and oxidative fibers by MEF2 and myofiber identity through ubiquitination and degradation of Class II HDACs is provided in **Figure 3**.

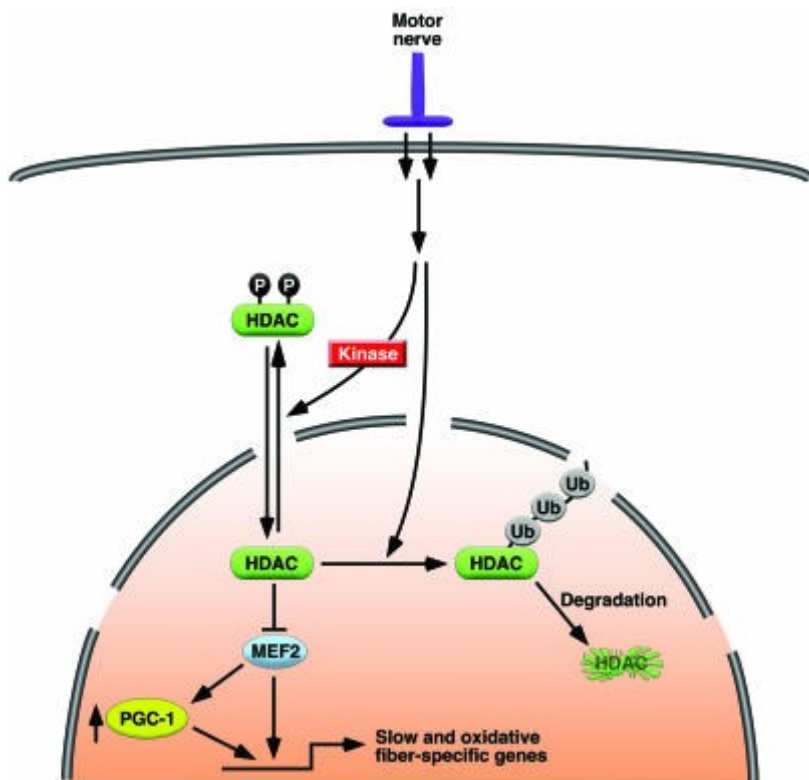


Figure 3. A model for the control of slow and oxidative fibers by MEF2 and class II HDACs (Pandorf et al., 2009).

Transcriptionally active genes are known to be associated with acetylation of histones (H3ac) and also trimethylation of lysine 4 in histone H3 (H3K4me3). Acetylated H3 (H3ac) and methylated H3 (H3K4me3) varied directly with the transcriptional activity of the MyHC genes in fast fiber-type plantaris and slow fiber-type soleus muscles. In this

study, muscle unloading resulted in a shift to fast MyHC gene expression in slow muscles and an increase in deacetylation of histone H3 at the MHC-I gene as well as the down-regulation of MyHC-I. Also, fast type IIX and IIB MyHCs were up-regulated with enhancement of H3ac. Enhancement of H3K4me3 is increased at the type IIX and IIB MHC with muscle unloading. Loss of H3ac or H3K4me3 was not associated with down-regulation of MyHC-IIA.

2.5 Syndromes Associated with Defects in Histone Deacetylase and Histone Acetyltransferase

The defects in histone deacetylase and histone acetyltransferase are associated with congenital disorders which include craniofacial malformation. Defects in HDAC4 are the cause of brachydactyly-mental retardation syndrome (BDMR), which resembles the anomalies found in Albright hereditary osteodystrophy. Common features are mild facial dysmorphism, congenital heart defects, distinct brachydactyly type E, mental retardation, developmental delay, seizures, autism spectrum disorder, and stocky build. Soft tissue ossification is absent without abnormalities in parathyroid hormone or calcium metabolism.

According to Kraft et al. (2011), haploinsufficiency of the histone acetyltransferase monocytic leukemia 4 (MYST4) gene causes Noonan syndrome–like phenotype. A balanced chromosomal translocation of MYST4 gene is seen in a patient with clinical features resembling Noonan syndrome. Noonan syndrome is a common monogenic condition characterized by postnatal reduced growth, cardiac defects and distinctive craniofacial dysmorphism. Noonan syndrome is associated with open bite.

Based on a MYST4-knock-out-mouse model, the study demonstrated that H3 acetylation plays a crucial role for neural, craniofacial, and skeletal morphogenesis by specifically regulating the MAPK signaling pathway (Kraft et al., 2011).

CHAPTER 3

AIMS OF THE INVESTIGATION

The study proposes to investigate expression of histone deacetylase 4 (HDAC4) and histone acetyltransferase 4 (MYST4) genes in masseter muscle of vertical dimension malocclusion, open bite and deep bite, and sagittal dimension malocclusion, Classes I, II and III, patients to test the hypothesis that there may be a relationship between chromatin modifying enzymes and fiber-type contractile proteins in various occlusal types. The aim can be further divided as the following:

1. Observe the differences in the relative quantity of HDAC4, relative quantity of MYST4, percent gene expression of MyHC and fiber type percent occupancy among the occlusal groups and subgroups.
2. Determine the correlation between the percent gene expression of MyHC of various fiber types to HDAC4 and MYST4 of various occlusal types.
3. Determine the correlation between the percent occupancy of each fiber types to HDAC4 and MYST4 of various occlusal types.

CHAPTER 4

MATERIALS AND METHODS

4.1 Muscle Samples

Masseter muscle samples used here had been obtained from subjects undergoing orthognathic surgery for treatment of malocclusion at the University of Lille, France. The subjects were Caucasian with mean age of 20 years old. A summary of subjects from whom muscle was obtained is shown on **Table 1**. Specimens were acquired as described previously (Rowlerson et al., 2005). Malocclusion classifications were based upon jaw dimension repositioning that was specified in the surgical treatment plan. The vertical and sagittal dimensions are given by the surgeons as the following: skeletal Class I, skeletal Class II, skeletal Class III, open bite, deep bite and normal bite. The diagnosis also includes the presence or absence of skeletal asymmetry. Based on the vertical dimension, the subjects were divided into 6 subgroups, deep bite with Class II (D2), deep bite with Class III (D3), normal bite with Class II (N2), normal bite with Class III (N3), open bite with Class II (O2) and open bite with Class III (O3), as shown in **Table 2**. Consent for subject participation was obtained according to regulations for ethical research by committees at the University of Lille and the Institutional Review Board of Temple University. Masseter samples were consistently taken at a position 1.5 cm from the lowest point of the mandible's angle during bilateral sagittal split osteotomy procedures and stored at -80°C before being processed for histologic analysis and isolation of RNA. RNA from one of each human forearm extensor, trapezius, quadriceps and triceps/deltoid limb muscles had been obtained from orthopedic surgeries during

earlier studies with approval (University of Pittsburgh and King’s College, London) and banked for comparative use with masseter muscle studies. **APPENDIX A.**

Table 1. Distribution of Subject Based on Gender and Age

Gender	Subjects (n)	Mean Age (yrs)	Vertical		Sagittal	
			Dimension	(n)	Dimension	(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

Table 2. Distribution of Subjects Based on the Occlusal Subgroups

Horizontal dimension	<i>Vertical Dimension</i>			
	Normal bite (n)	Open bite (n)	Deep bite (n)	Total (n)
Class II	7	12	6	25
Class III	5	7	5	17
Total	12	19	11	42

4.2 RNA Isolation & Real Time PCR

Muscle RNA was isolated with TRIzolTM reagent (Invitrogen, Carlsbad, CA), digested with DNase I, re-isolated with RNAqueous® and quantified by absorbance at

A₂₆₀ as described previously (Horton, 2008). RNA for the HDAC4 and MYST4 genes was quantified in 43 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 HDAC4 (Hs01041648_m1), MYST4 (Hs00202463_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 residual 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 was used in all assays as shown on **Table 3**.

Table 3. Standard conditions for RT-PCR

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

Initial RT-PCR assays used serial dilutions of skeletal muscle RNA to show that amplification efficiencies of HPRT1 endogenous control and HDAC4 and MYST4 were within 10% of one another (**Figure 4**). 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 ($\Delta\Delta C_T$) (Livak and Schmittgen, 2001) that measures fold-difference between normalized amounts of target in test samples.

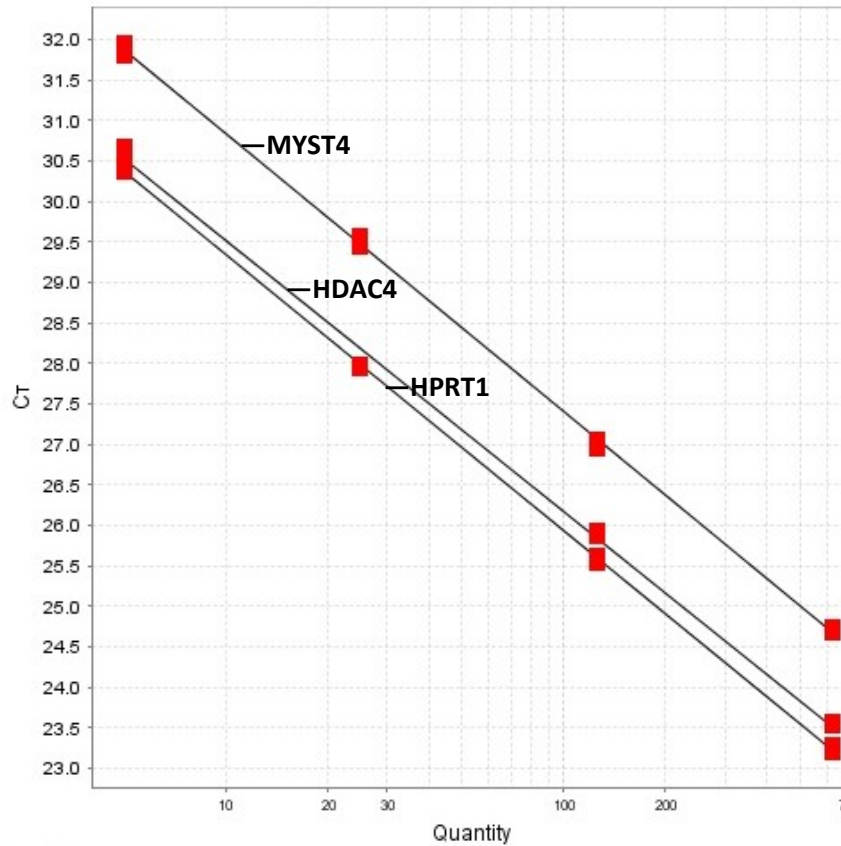


Figure 4. Comparison of RT-PCR amplification plots of HDAC4, MYST4 and HPRT1 using standard quantities of 5ng, 25ng, 125ng and 625ng a commercial human skeletal muscle RNA.

An internal reference when both the reference and target genes are amplified at approximately the same efficiency. The relative quantity (RQ) of expressed RNA for HDAC4 and MYST4 is calculated using the equation:

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

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

ΔC_T (Muscle Sample) = C_T (Target Gene in Biopsy Muscle) - C_T (HPRT1 in Biopsy Muscle)

ΔC_T (Reference) = C_T (Target Gene in Muscle Standard) - C_T (HPRT1 in Muscle Standard)

Thus, measurements of HDAC4 and MYST4 are normalized to HPRT1. Then, RQ values are determined by comparing the normalized HDAC4 or MYST4 quantity in each sample of masseter or limb muscle to the normalized HDAC4 or MYST4 quantity in the reference sample of skeletal muscle RNA.

4.3 Analysis of Fiber type Properties

Fiber type area and percent occupancy had been determined previously for 42 of 43 masseter muscle samples used in this study (Rowlerson et al., 2005). Percent occupancy was determined as the percent of muscle area occupied by a fiber type using immunohistochemistry stains. 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 prepared by Dr. Anthea Rowlerson) and α -cardiac (MAS 366). Eight fiber types were identified using the immunostaining of masseter muscle with this set of antibodies. These eight fiber types were organized into 4 groups as the following: 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). Only the fibers with adequate staining and morphology were included and damaged fibers were rejected for analysis. Occupancy for each of the 4 fiber groups was calculated by multiplying fiber areas and number of fiber. Then, percent occupancy of total occupancy was measured for each fiber types. For the present study mean percent occupancy values for the fiber types were calculated and this data used to analyze correlations to HDAC4 and MYST4 expression by both sagittal and vertical malocclusion classes.

Expression of genes for MyHC-I/ β , IIA, IIX, perinatal (neonatal) and α (atrial), in another 42 of the 43 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 HDAC4 and MYST4 expression.

4.4 Statistical Analyses

Descriptive statistics including mean and standard deviation were calculated to analyze the data set by sex, gender, vertical and sagittal malocclusion groups and subgroups based on vertical and sagittal malocclusion. The subjects were divided into open bite and deep bite based on the vertical dimension, and into Class II and III based on the sagittal dimension. Then, the subjects were further divided into the following

subgroups: deep bite with Class II (D2), deep bite with Class III (D3), normal bite with Class II (N2), normal bite with Class III (N3), open bite with Class II (O2) and open bite with Class III (O3).

A generalized linear model was created consisting of the variables, sex, age, vertical malocclusion groups, sagittal malocclusion groups HDAC4 and MYST4 expression, to predict HDAC4 RQ and MYST4 RQ. A second model was also created consisting of the six occlusal subgroups to predict HDAC4 and MYST4 expression. Generalized linear model is used when flexibility in data structure is necessary as it can accommodate nominal, ordinal, continuous and discrete data. Two one-way ANOVA models that ignore the effects of age and sex were created.

The final 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 HDAC4 expression and both MyHC 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). Then, Kendall's Tau-b test was repeated to examine the relationship between MYST4 expression and both MyHC gene expression and fiber type percent occupancy.

CHAPTER 5

RESULTS

5.1 Aim 1- General Comparison of RQ Among Occlusal Subgroups

Following the RT-PCR, the gene expressions of HDAC4 and MYST4 were measured as relative quantity (RQ) as shown in the **Table 4 and 5**. Since only one subject was available for Class I, the Class I subject was excluded from the comparison. A complete set of HDAC4 and MYST4 data can be found in **APPENDIX B**.

Table 4. HDAC4 RQ by Vertical and Sagittal Dimensions

Vertical		Sagittal	
		2	3
Deep Bite	Mean	1.06	0.96
	SD	0.49	0.47
Normal Bite	Mean	0.68	1.20
	SD	0.37	1.23
Open Bite	Mean	0.79	1.96
	SD	0.45	1.78

Table 5. MYST4 RQ by Vertical and Sagittal Dimension

Vertical		Sagittal	
		2	3
Deep Bite	Mean	3.30	4.11
	SD	0.88	1.75
Normal Bite	Mean	3.04	4.28
	SD	1.47	1.16
Open Bite	Mean	3.28	4.85
	SD	1.19	1.78

Greater RQ expression levels of HDAC4 and MYST4 were observed for subjects with Class III when compared to the subjects with Class II as shown on **Figure 5**.

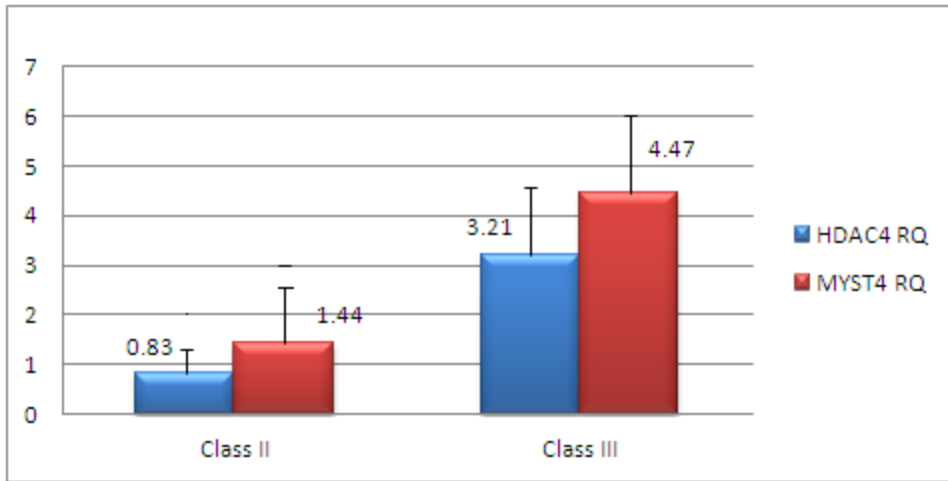


Figure 5. HDAC4 and MYST4 RQ by Sagittal Dimension

5.2 Aim 1- Percent Occupancy Among Occlusal Subgroups

Percent occupancy data for the masseter samples can be found on **APPENDIX C**. Type I percent occupancy was compared among each subgroup as shown in **Figure 6** and **Table**

6. O2 and O3 groups had higher type I percent occupancy when compared to D2 and D3 groups respectively. However, the differences were statistically insignificant.

Table 6. Percent Occupancy of Type 1 Fiber

Vertical		Sagittal	
		2	3
Deep Bite	Mean	46.11	37.84
	SD	12.00	17.72
Normal Bite	Mean	47.32	45.66
	SD	18.73	18.36
Open Bite	Mean	60.45	44.58
	SD	15.04	16.48

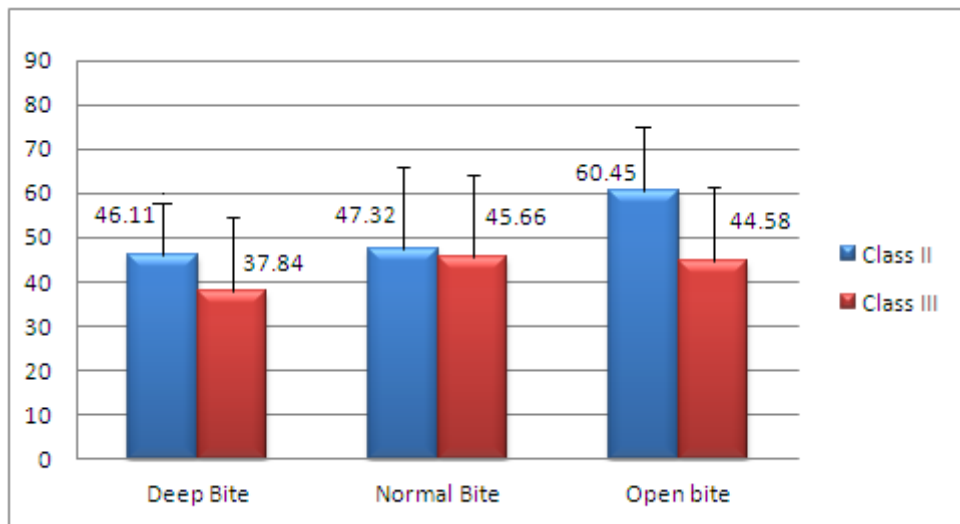


Figure 6. Percent Occupancy of Type 1 Fiber

No statistically significant differences were found for Hybrid I/II fiber percent occupancy as shown on **Table 7**.

Table 7. Percent Occupancy of Hybrid I/II

Vertical		Sagittal	
		2	3
Deep Bite	Mean	21.70	24.92
	SD	20.15	21.43
Normal Bite	Mean	33.86	35.64
	SD	14.11	17.16
Open Bite	Mean	29.95	30.67
	SD	16.57	22.01

An increased level of type II percent occupancy was observed for deep bite when compared to that of normal and open bite subjects as shown on **Table 8** and **Figure 7**. Class III subjects showed greater percent occupancy of type II fiber when compared to the Class II subjects with respective vertical classification. However, the differences were statistically insignificant.

Table 8. Percent Occupancy of Type II Fiber

Vertical		Sagittal	
		2	3
Deep Bite	Mean	25.48	32.76
	SD	12.22	17.72
Normal Bite	Mean	3.16	8.23
	SD	3.92	2.77
Open Bite	Mean	3.40	11.38
	SD	5.98	25.87

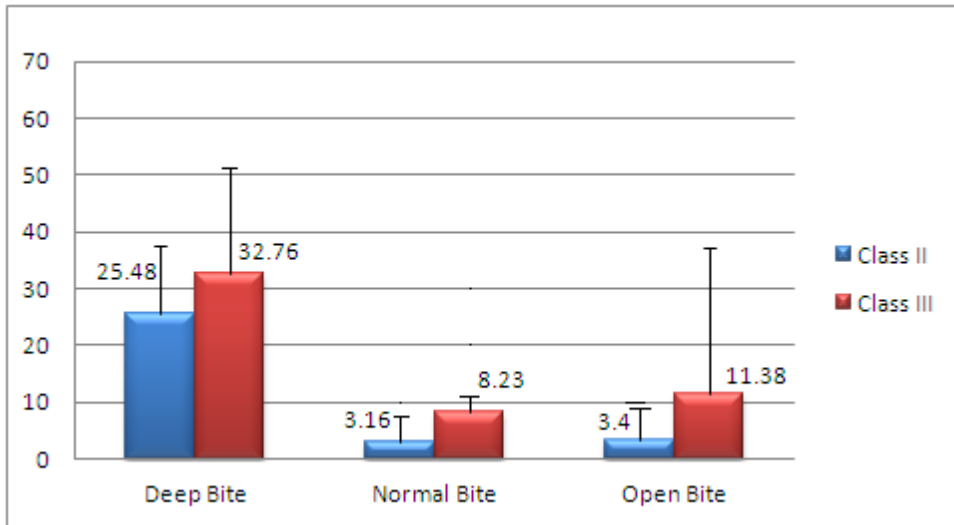


Figure 7. Percent Occupancy of Type II Fiber

No discernible trend could be found among the subgroups on the neonatal/atrial fiber percent occupancy as shown on **Table 9**.

Table 9. Percent Occupancy of Neo/Atrial Fiber

Vertical		Sagittal	
		2	3
Deep Bite	Mean	6.69	4.47
	SD	4.89	2.79
Normal Bite	Mean	15.64	10.46
	SD	18.21	6.13
Open Bite	Mean	6.18	13.35
	SD	6.96	10.37

5.3 Aim 1- Percent Gene expression of MyHC Among Occlusal Subgroups

The percent expression of MyHC type I fiber was compared among each subgroup as **Table 10** and **Figure 8**. Greater percent expression of MyHC type I fiber was observed for open bite subjects when compared to deep bite subjects with respective sagittal dimension. The percent expression of MyHC I fiber was increased for Class III subjects when compared to that of Class II subjects with respective vertical classification. However, the difference was statistically insignificant. Percent MyHC data for the masseter samples can be found on **APPENDIX D**.

Table 10. Percent Gene Expression of MyHC I

Vertical		Sagittal	
		2	3
Deep Bite	Mean	48.77	36.56
	SD	12.22	18.86
Normal Bite	Mean	47.70	63.99
	SD	25.77	10.98
Open Bite	Mean	61.31	51.44
	SD	16.18	24.42

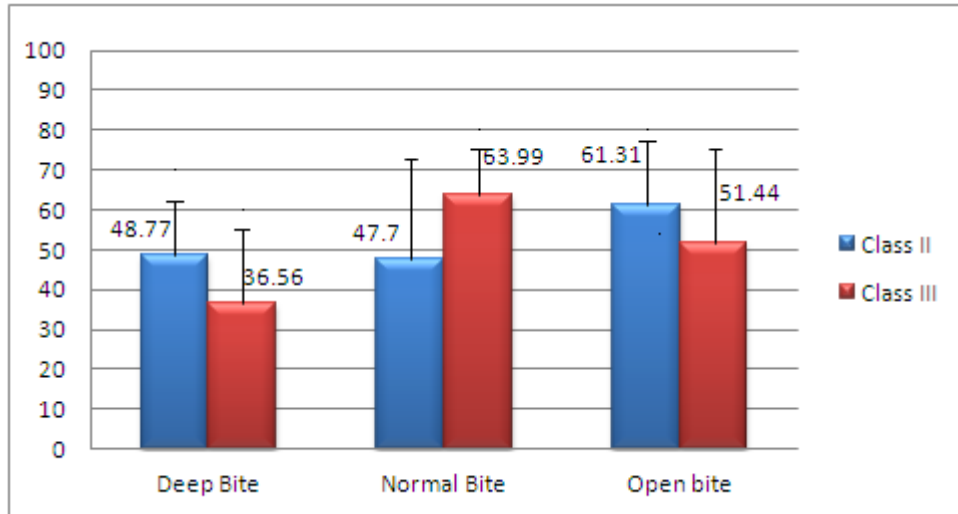


Figure 8. Percent Gene Expression of MyHC I

The percent expression of MyHC IIA/IIX fiber was compared among each subgroup as **Table 11** and **Figure 9**. A greater percent expression of MyHC IIA/IIX fiber was observed for deep bite subjects when compared to the open bite subjects with respective sagittal dimension. However, the difference was statistically insignificant. Observable trend was not seen between the Class II and Class III on percent expression of MYHC type IIA/IIX fibers.

Table 11. Percent Gene Expression of MyHC IIA/IIX

Vertical		Sagittal	
		2	3
Deep Bite	Mean	25.65	52.04
	SD	20.22	19.75
Normal Bite	Mean	20.03	15.56
	SD	15.83	8.70
Open Bite	Mean	21.14	22.77
	SD	17.83	20.90

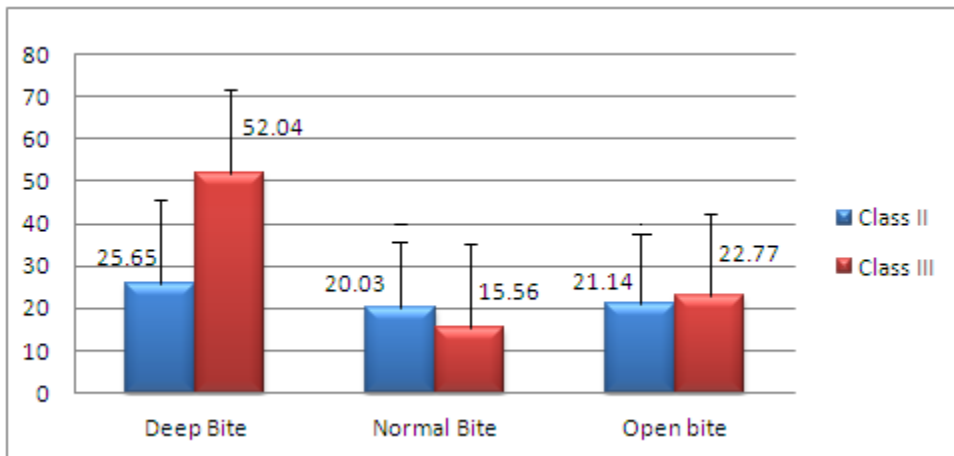


Figure 9. Percent Gene Expression of MyHC IIA/IIX

Percent expression of MyHC for neonatal and atrial genes was compared among each subgroup on **Table 12, Figure 10 Table 13 and Figure 11**. No significant trend was found for the neonatal or atrial fibers among various occlusal types.

Table 12. Percent Gene Expression of Neonatal MyHC

Vertical		Sagittal	
		2	3
Deep Bite	Mean	7.13	1.68
	SD	7.81	1.09
Normal Bite	Mean	9.66	8.13
	SD	4.83	6.19
Open Bite	Mean	7.76	7.08
	SD	6.47	6.14

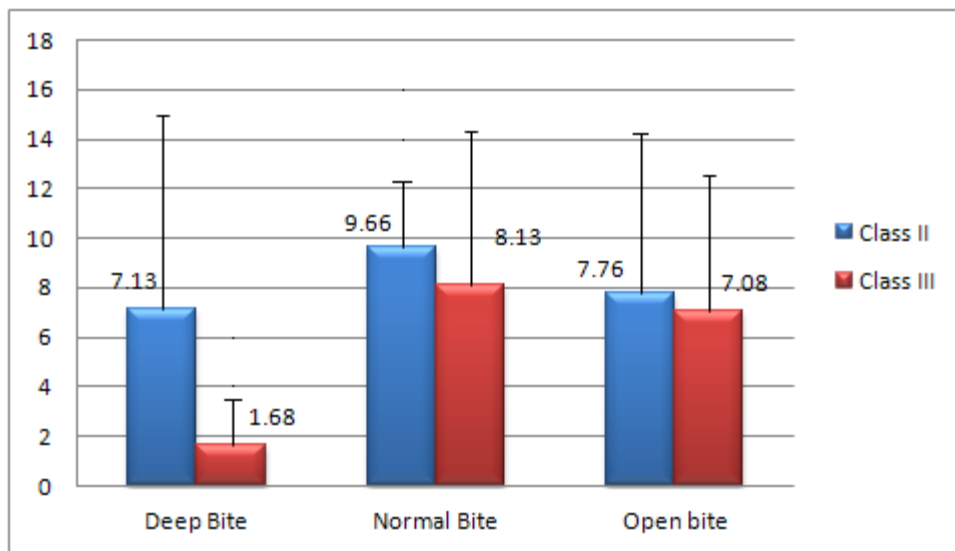


Figure 10. Percent Gene Expression of Neonatal MyHC

Table 13. Percent Gene Expression of Atrial MyHC

Vertical		Sagittal	
		2	3
Deep Bite	Mean	18.43	9.70
	SD	9.89	4.29
Normal Bite	Mean	22.59	12.29
	SD	14.83	12.49
Open Bite	Mean	9.77	18.69
	SD	5.37	26.56

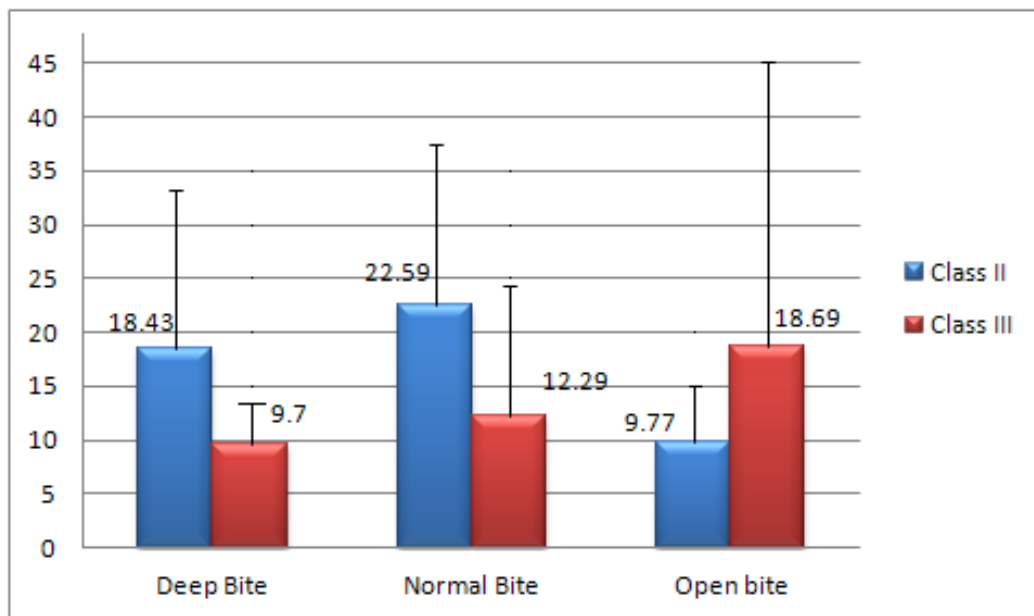


Figure 11. Percent Gene Expression of Atrial MyHC

5.4 Aim 2- Correlations HDAC4 to Percent Occupancy

Significant correlations were found using Kendall's tau-b to analyze the relationship between HDAC4RQ and MYST4RQ and percent occupancy of various occlusal types. Furthermore, the correlations between HDAC4 and MYST4 and percent MyHC of various occlusal types were also significant using Kendall's tau-b analysis. 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 the most appropriate test. Summaries of the correlations for the chromatin modifying genes and subgroups with percent occupancy of occlusal subgroups are provided in **Table 14** and **Table 15**. Plots of highly correlated values between the chromatin modifying enzymes and percent occupancy were provided in **APPENDIX E**.

Table 14. Correlations HDAC4RQ to Percent Occupancy of Fiber Types

Group	Type 1 %Occ	Hybrid I/II %Occ	Type II %Occ	Type N/A %Occ
D2	0.80*	-0.80*	-0.20	0.20
D3	0.00	0.00	0.40	0.40
N2	-0.52	0.33	-0.04	0.04
N3	-1.00*	0.66*	0.33	0.33
O2	-0.02	-0.15	0.37	0.15
O3	-0.23	-0.14	0.23	-0.71*
Total	-0.24	-0.04	0.08	0.04

* Statistically significant values (> 0.06 or <-0.06)

Table 15. Correlations MYST4RQ to Percent Occupancy of Fiber Type

Group	Type 1 %Occ	Hybrid I/II %Occ	Type II %Occ	Type N/A %Occ
D2	-0.20	0.20	0.00	-0.40
D3	-0.20	0.20	0.20	0.20
N2	-0.42	0.23	0.42	0.52
N3	-0.66*	1.00*	0.00	0.00
O2	0.15	-0.24	0.11	0.06
O3	-0.09	0.14	0.15	0.55
Total	-0.16	0.05	0.05	0.06

* Statistically significant values (> 0.06 or <-0.06)

Correlation values greater 0.06 or less than -0.60 were considered significant. Significant correlations were found between HDAC4 RQ to the percent occupancies of type I and type II fiber in D2, to the percent occupancies of type I fiber in N3 and to the percent occupancy of N/A fiber in O2. The relationship between MYST4 and the percent occupancy of type I and hybrid I/II fiber in N3 showed significant correlation.

HDAC4 RQ of Class II deep bite was positively correlated to type 1 percent occupancy and negatively correlated to hybrid I/II percent occupancy and type II percent occupancy as shown in **Figure 12**.

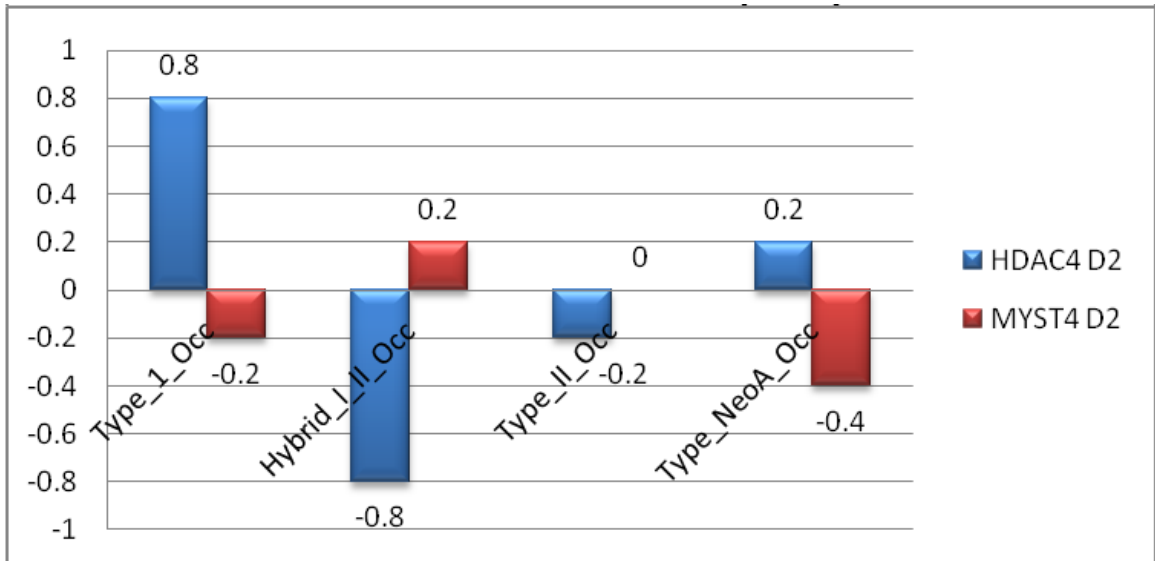


Figure 12. Correlation of HDAC4 and MYST4 of Deep bite Class II to Percent Occupancy

HDAC4 RQ of O3 was negatively correlated to neonatal/atrial fiber percent occupancy while MYST4 RQ of O3 was positively correlated to neonatal/atrial fiber percent occupancy. However, the correlation value of MYST4 was not statistically significant for either open bite or deep bite subjects.

5.5 Aim 3- Correlations HDAC4 to Percent MyHC Gene Expression

Significant correlations were found using Kendall's tau-b to analysis the relationship between HDAC4 RQ and MYST4 RQ and percent MyHC expressions of various occlusal types. Summaries of the correlations for the chromatin modifying genes and subgroups with various percent MyHC types are provided in **Table 16** and **Table 17**.

Table 16. Correlations HDAC4RQ with Percent MyHC Gene Expression

Group	I	IIA	IIX	IIAIIIX	Neo	Atrial	NA
D2	0.20	-0.06	0.06	-0.06	0.06	-0.60*	-0.33
D3	-0.67*	0.66*	0.00	0.66*	-0.33	0.33	0.33
N2	-0.73*	0.46	0.87*	0.73*	0.06	0.33	0.33
N3	-0.20	-0.20	0.20	0.00	1.00*	0.00	0.40
O2	-0.34	0.05	0.45	0.13	0.02	0.31	0.09
O3	0.20	0.60*	-0.06	0.33	-0.33	-0.60*	-0.60*
Total	-0.24	0.18	0.21	0.23	0.05	-0.05	-0.03

* Statistically significant values (> 0.06 or <-0.06)

Table 17. Correlations MYST4RQ with Percent MyHC Gene Expression

Group	I	IIA	IIX	IIAIIIX	Neo	Atrial	NA
D2	-0.20	-0.20	-0.20	-0.20	0.46	0.06	0.33
D3	-0.33	1.00*	-0.33	0.33	0.00	0.00	0.00
N2	-0.33	0.06	0.20	0.06	0.46	0.46	0.73*
N3	0.00	0.40	0.00	0.20	0.40	-0.20	-0.20
O2	0.30	-0.49	0.01	-0.38	0.01	-0.05	-0.05
O3	-0.06	-0.46	-0.60*	-0.73*	0.73*	0.20	0.20
Total	-0.06	0.01	-0.08	-0.06	0.14	-0.06	0.03

* Statistically significant values (> 0.06 or <-0.06)

Correlation values greater 0.06 or less than -0.60 were considered significant. Significant correlations were found between HDAC4 RQ to the percent occupancies of atrial fiber in D2, to the percent occupancies of type I, IIA and IIA/IIX fiber in D3, to the percent occupancies of type I, IIX and IIA/IIX fiber in N2, to the percent occupancy of neonatal fiber in N3 and to the percent occupancy of IIA, atrial and N/A fiber in O3. The relationship between MYST4 RQ and the percent gene expression of MyHC IIA in D3 showed significant correlation as well as the relationship between MYST4 RQ and the percent gene expression of MyHC N/A in N2, and to the percent gene expression of MyHC IIA, atrial and N/A fiber in O3. Plots of highly correlated values between the chromatin modifying enzymes and percent MyHC level were provided in **APPENDIX F**.

The correlation between HDAC4 RQ and percent MyHC expression of various fibers of deep bite subjects are summarized in **Figure 13**. MyHC atrial fiber expression had a strong negative correlation to HDAC4 RQ of deep bite Class II group. HDAC4 of Class III deep bite group had strong positive correlation with expression of MyHC IIA and IIX fiber and strong negative correlation with MyHC I and Atrial fibers.

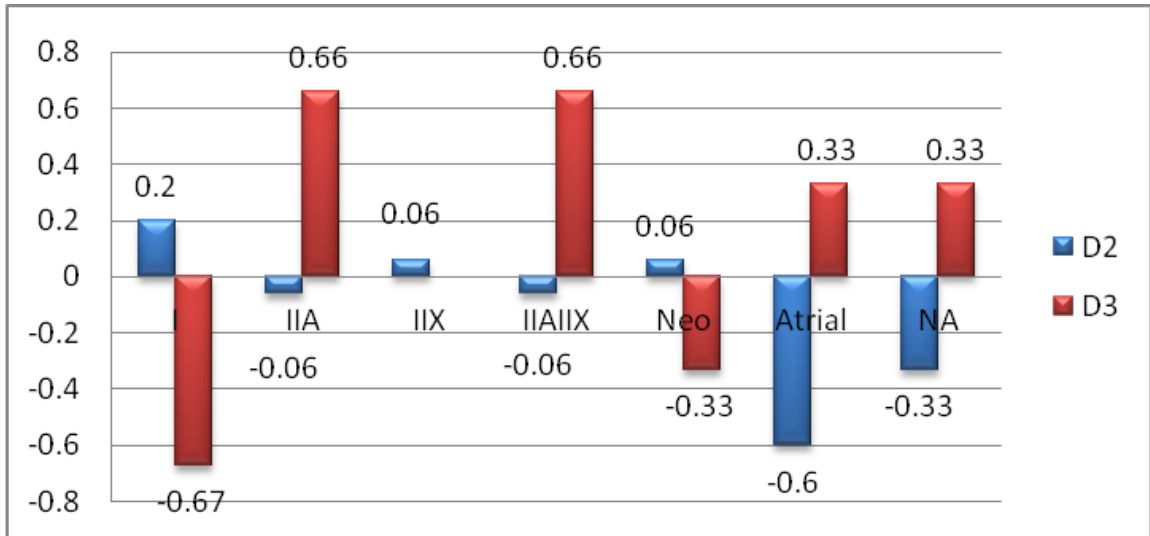


Figure 13. Correlations HDAC4 RQ and Percent MyHC Gene Expression in Deep Bite Subject

Correlations of HDAC4 RQ and percent MyHC in open bite subjects are summarized as

Figure 14. MyHC atrial fibers had strong negative correlation to MYST4 RQ of open bite Class III group. HDAC4 RQ of Class III open bite and MYST4 of Class III open bite had strong positive correlation to MyHC II. MYST expression of Class III open bite was correlated negatively to MyHC IIA and IIX, and positively to MyHC neonatal fibres.

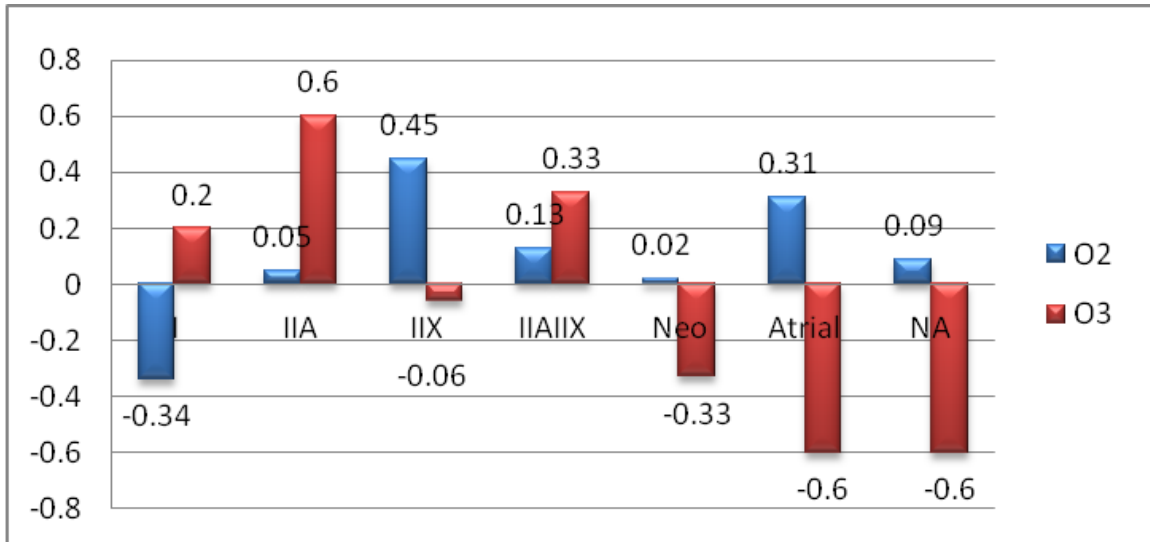


Figure 14. Correlations HDAC4 RQ and Percent MyHC Gene Expression in Open Bite

Correlations of HDAC4 RQ and percent MyHC in Class III subjects are summarized as **Figure 15**. Positive correlation was found between HDAC4 expression of Class III with both open and deep bites. Only HDAC4 of D3 had negative correlation with the expression of MyHC I fiber.

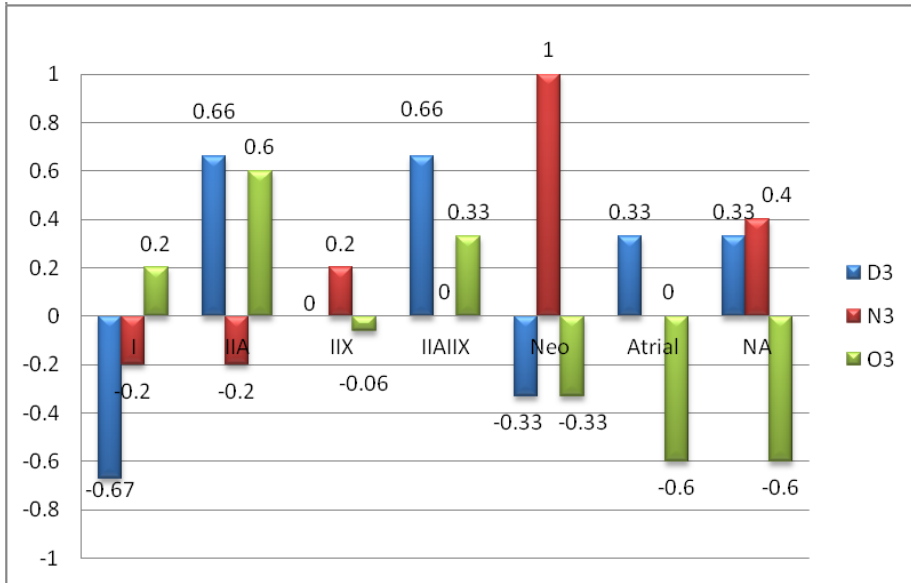


Figure 15. Correlations HDAC4 RQ to Percent MyHC Gene Expression in Class III

The expression of MyHC IIA had strong correlations to HDAC4 RQ of both D3 and O3 while the expression of MyHC I had strong correlations to HDAC4 RQ of both D3 and O3 as seen in **Figure 16**.

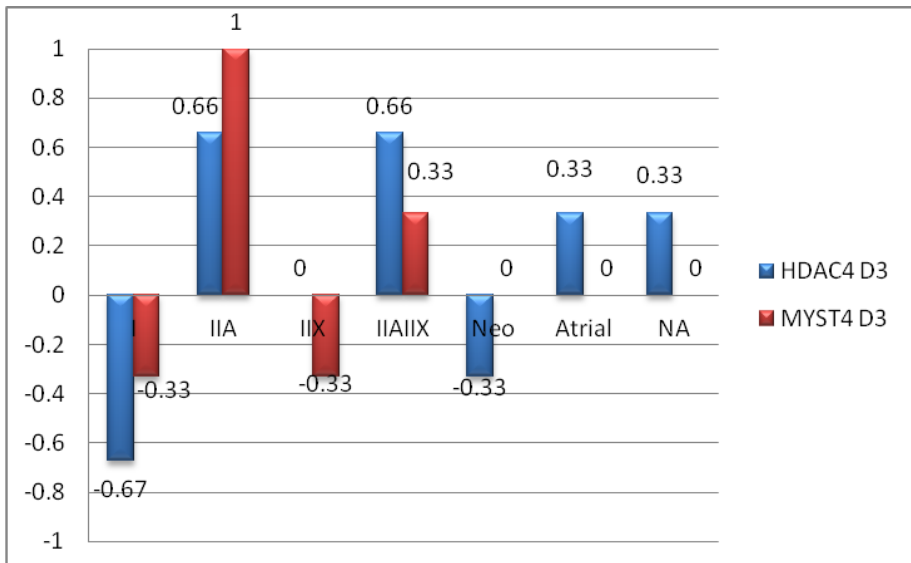


Figure 16. Correlations of HDAC4 RQ and MYST4 RQ to Percent MyHC Gene Expression in Deep Bite Class III

An inverse relationship was found between HDAC4 and MYST4 in the correlation analysis of percent MyHC in open bite class III subjects as seen in **Figure 17**.

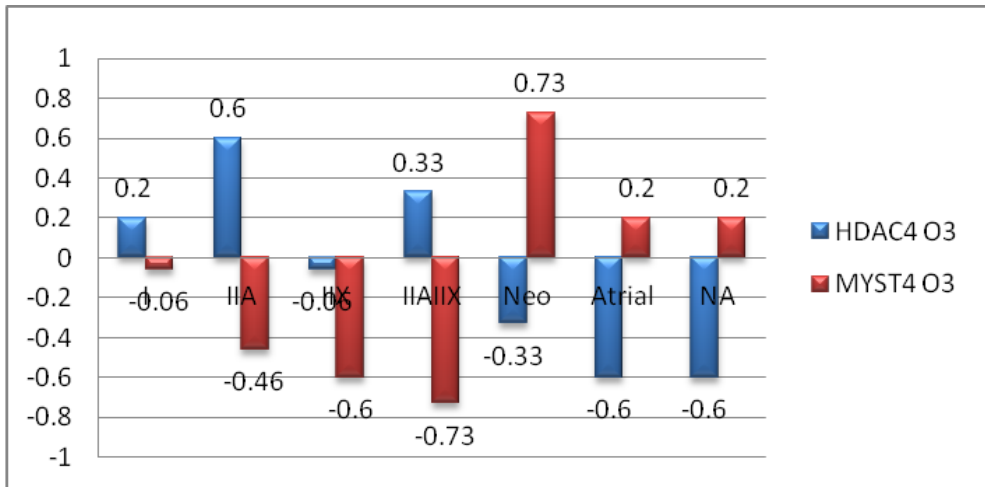


Figure 17. Correlations HDAC4 RQ and MYST4 to Percent MyHC Gene Expression in Open Bite Class III

5. 6. Statistical Analysis

Power analysis was performed as shown on **Table 18**. Sample size of 108 or 18 per group is needed to get accurate HDAC4 RQ. Power was 80%, and partial Eta Squared was .0112 for HDAC4.

Table 18. HDAC4RQ Percent Occupancy Power Analysis

Partial Eta Squared	0.112
Effect Size	0.355
Alpha (error probability)	0.05
Power	80%
Number of Groups	6
Sample Size	108 or 18 Per group
Study Power	30%

Power analysis was performed as shown on **Table 19**. Sample size of 102 or 17 per group is needed to attain an accurate estimation of MYST4 RQ significance. Power was 80%, and partial Eta Squared was .0121 for HDAC4.

Table 19. MYST4RQ Percent Occupancy Power Analysis

Partial Eta Squared	0.121
Effect Size	0.371
Alpha (error probability)	0.05
Power	80%
Number of Groups	6
Sample Size	102 or 17 Per group
Study Power	32%

Power analysis was performed as shown on **Table 20**. Sample size of 102 or 16 per group is needed to get accurate HDAC4 RQ. Power was 80%, and partial Eta Squared was .0126 for HDAC4. The power analysis indicate that MYHC data require less sample size than % occupancy data to accurately measure HDAC4 RQ significance, indicating that MYHC data is slightly more reliable than the percent occupancy data.

Table 20. HDAC4RQ MYHC Power Analysis

Partial Eta Squared	0.126
Effect Size	0.379
Alpha (error probability)	0.05
Power	80%
Number of Groups	6
Sample Size	96 or 16 Per group
Study Power	34%

Power analysis was performed as shown on **Table 21**. Sample size of 72 or 12 per group is needed to get accurate evaluation of MYST4 RQ significance. Power was 80%, and partial Eta Squared was .0163 for MYST4.

Table 21. MYST4RQ MYHC Power Analysis

Partial Eta Squared	0.163
Effect Size	0.441
Alpha (error probability)	0.05
Power	80%
Number of Groups	6
Sample Size	72 or 12 Per group
Study Power	45%

No significant differences were found using generalized linear models consisting of the variables sex, age, vertical (deep, normal and open bite) and sagittal (Class II and III) to predict HDAC4 RQ and MYST4 RQ as seen in **APPENDIX G**. No significant differences were found using generalized linear models consisting of individual variables of sex, age, subgroups to predict HDAC4 RQ and MYST4 RQ. No group differences were found using two one-way ANOVA models that ignore the effects of age and sex as seen in **APPENDIX H**.

CHAPTER 6

DISCUSSION

6.1 Microarray Data of Masseter Muscle

In a single analysis of microarray data comparing masseter muscle gene expression in one open bite subject to one deep bite subject, HDAC4 was 6.96 fold greater in deep bite. This high level of HDAC4 may be associated with the decrease in MyHC I gene expression by inactivating the chromatin segment that codes for MyHC type 1, which is -1.37 fold less in deep bite (Horton M. unpublished results). Gene expression of MYST4 was 2.57 fold higher in deep bite subjects compared with open bite subjects. Greater gene expression was observed for myosin IIA (6.54 fold), and IIX (13.74 fold) when a deep bite subject was compared to a deep bite subject suggesting that increased MYST4 in open bite subjects may affect gene expression of the fast-type myosins.

6.2 Sagittal Dimension Comparison

Greater HDAC4 RQ and MYST4 RQ were observed for Class III subjects when compared to that of Class II subjects with the respective vertical classification. Increased expression of HDAC4 and MYST4 in Class III subjects indicates that these genes may have an effect on the length of mandible. As both HDAC4 and MYST4 increased in Class III subjects, HDAC4 and MYST4 may not have an antagonistic role in muscle fiber differentiation of masseter muscle. HDAC4 catalyzes the removal of acetyl groups from lysine residues in histones and non-histone proteins, resulting in transcriptional

repression. MYST4 can negatively regulate transcription MYST4 by activating transcription of inhibitor for muscular fiber differentiation. Therefore, it is possible for MYST4 and HDAC4 to have functions that are not antagonistic. In part, these functions may be the result of different intracellular locations where HDAC4 and MYST4 are found (De Ruijter et al., 2003). HDAC4 shuttles between the nucleus and cytosol while MYST4 is largely found in the nucleus. Therefore, the two genes may be involved in two distinct pathways. For example, HDAC4 may have a role in the formation of Class III malocclusion through muscle fiber differentiation involving MEF2. On the other hand, MYST4 may have role in the formation of mandibular prognathism through mechanisms involving Runt-related transcription factor-2 (RUNX2). RUNX2 is a key transcription factor associated with osteoblast differentiation and tooth formation whose transcriptional activation is MYST4 dependent. MYST4 is required for RUNX-2 dependent transcriptional activation. RUNX2 is reported to play a role in condylar growth during early chondrocytic differentiation and maturation (Rath-Deschner et al., 2010). A RUNX2 knock-out mice model showed decreases in corpus length, gonial angle and size of the mandible (Rath-Deschener et al., 2010). MYST4 may favor development of Class III and mandibular prognathism as a result of interaction with RUNX2 by influencing the osteoblastic and chondrocytic development of mandibular structures. To make the analysis more complex, MYST4 may be involved in more than one pathway to regulate the craniofacial growth. Based on MYST4-knock-out-mouse model, H3 acetylation plays crucial role for neural, craniofacial, and skeletal morphogenesis by specifically regulating the MAPK signaling pathway (Kraft et al., 2011).

Previous studies found no differences in fiber type characteristics between Class II and Class III subjects (Sciote, 2012; Shaughnessy et al., 1989). However, increased levels of MyHC type I fibers and decreased levels of MyHC type IIX and IIA fibers were found in Class II subjects when compared to that of Class III subjects with respective vertical classification. Type I fiber percent occupancy also increased and type IIA fiber percent occupancy decreased in Class II subjects when compared to the Class III subjects with respective vertical malocclusion. Based on our data, individuals with retrusive mandible tend to have higher composition of type I fibers while individuals with protrusive mandible tend to have a higher composition of type II fibers. Our finding is supported by a competitive PCR study by Gedrange et al. (2005) who found higher MyHC type I amounts in the anterior masseter muscle in 10 patients with preoperative mandibular retrognathia having the same sagittal discrepancies as those patients with prognathia. Sagittal dimension malocclusion has multifactorial etiology, and the degree of influence by muscle function is difficult to determine. Further study is needed to gain better understanding of the antero-posterior development of craniofacial structure and muscle fiber phenotype.

6.3 Vertical Dimension Comparison

Open bite subjects had greater type 1 fiber percent occupancy when compared to the deep bite subjects with respective sagittal classification. Class II and Class III subjects with open bite had higher type I fiber occupancy when compared to the Class II and Class III subjects with deep bite respectively. Furthermore, greater percent expression of MYHC type I fibers was observed when O2 and O3 groups were compared to D2 and D3

respectively. In a series of studies from Rowlerson et al. (2005) and Sciote et al. (2012), increased levels of type I fiber percent occupancy is found in open bite subjects. The current data suggest that decrease in occlusal contact area is associated with increase in level of slow twitch type I fibers which are suitable for postural function. Greater percent occupancy was observed for fast twitch type II fibers in deep bite subjects when compared to that of normal and open bite subjects. Greater percent expression of MyHC type II (IIA and IIX) fibers was observed for deep bite subjects when compared to the open bite subjects with respective sagittal dimension, confirming data from previous studies showing reductions in type II fiber in subjects with anterior open bites (Rowlerson et al., 2005; Suchak et al., 2009; Sciote et al., 2012). The functional characteristic of high but fatigable contractile force by type II fiber may be a factor in the development of deep bite.

6. 4 Correlation Comparison

A strong positive correlation was found between HDAC4 RQ of Class II deep bite, and type I percent occupancy, possibly indicating that HDAC may be repressing inhibitory pathways that favor development of Class II deep bite. As Class II deep bite is often associated with Class II division-2 malocclusion, the pathway involving the fiber type 1 phenotype and HDAC4 activity may favor the development of Class II division 2 which is characterized by mandibular retrognathism, retroclined maxillary incisors and low mandibular plane angle. A strong negative correlation was found between HDAC4 of Class III deep bite subjects and MyHC I fiber while a strong positive correlation was found between HDAC4 of Class III deep bite subjects and MyHC IIA and IIA/IIX. In

contrast to what might be expected, MYST4 also had a strong positive correlation with percent MyHC IIA expression in Class III deep bite masseter samples suggesting a potential relationship to genes for both acetylation and deacetylation. Clearly, additional data is needed to confirm this observation, requiring more masseter muscle samples from surgical patients in a future investigation.

Previous study has shown that the level of MyHC neonatal fiber decreases in human masseter muscle with increased age of the subject and may indicate a lower regenerative capacity of masseter muscle in older individuals (Cvetko et al., 2012). However, no correlation was found between age and fiber type classification in the present study.

6. 5 Limitation of the Study

Malocclusion has multifactorial and polygenic origin with a threshold for expression resulting from an interaction between susceptibility genes and environmental factors. The chromatin modifying genes studied in this experiment are two of many genes involved in the complex process in the formation of malocclusion and dentofacial deformities. For example, Class III is associated to genes that encode growth factors or other signaling molecules that are involved in condylar growth under mechanical strain. Variation in the expression level of Indian hedgehog homolog (IHH), parathyroid-hormone like hormone (PTH1H), insulin-like growth factor-1 (IGF-1), vascular endothelin growth factor (VEGF) seem to play an important role in the etiology of Class III malocclusion. (Xue et al., 2010) Furthermore, chromosomal loci 1p36, 12q23, and 12q13 seem to contain genes with increased susceptibility to Class III malocclusion (Xue

et al., 2010). Another important factor to consider is that HDAC4 is not the only Class II HDAC involved in slow muscle fiber differentiation. Studies with knockout animals revealed that HDAC4, -5, and -9 were able to compensate for each other's absence (Suzuki et al., 2007). Therefore, it is important to understand that HDAC4, MYST4 and fiber type composition does not cause a malocclusion exclusively.

Environmental influences on the formation of dentofacial deformity need to be considered when analyzing our data. For example, Ohnuki et al. (2009) indicated that the occlusal vertical dimension and myosin heavy chain composition in masseter and digastric muscle changed between freely moving control and bite-opened rats. Not only the duration of activity but the magnitude of muscular activity influences muscle fiber type composition. Soft diet facilitated early expression of IID/X and IIB isoforms, and a decline in the expression of neonatal and IIA isoforms in a rat model (Saito et al. 2002). Soft diet seems to facilitate an even more MyHC IIB-rich phenotype in the masseter muscle than a hard diet. Animal studies allow better control of the functional activity and environmental factors to minimize the confounding factors. For example, Pandorf et al. (2009) could limit activity of rats by suspending the limb when studying the effect of muscle unloading in soleus muscle. However, animal model data may not be reliable representations of the muscle fiber composition in masseter muscle in human, as differences in muscle properties exist between species. Since daily activity of the human subjects in the present study was neither known nor could be controlled, the data inherently contain confounding factors caused by the variation of masticatory activity among individuals.

The use of chromatin immunoprecipitation (ChIP) technique along with RT PCR in the future may allow better understanding of the histone modifications and the specific loci in the genome where they occur. Chromatin immunoprecipitation is a contemporary method to investigate the interaction between proteins and DNA in the cell. ChIP allows investigation of association between specific proteins and specific genomic regions, such as transcription factors on promoters or other DNA binding sites to possibly allow definition of involved cistromes. Data from chromatin immunoprecipitation of open bite and deep bite samples would provide a better understanding of the epigenetic phenomenon involving the chromatin modifying genes and musculoskeletal genes of interest.

CHAPTER 7

CONCLUSIONS

This is the first study, to our knowledge, to investigate the relationship between chromatin modifying enzymes and fiber-type contractile proteins in human masseter muscle of various occlusal types. Class III was associated with higher relative expression of HDAC4 and MYST4 when compared to that of Class II. Increased expression of HDAC4 and MYST4 in Class III subjects may indicate that these genes may have effect on the length of mandible. As both HDAC4 and MYST4 increased in Class III subjects, HDAC4 and MYST4 may not have antagonistic role in muscle fiber differentiation of masseter muscle, and the genes may be involved in more than one regulatory pathway for the formation of malocclusion. Based on our data, individuals with retrusive mandible tend to have higher composition of type I fibers while individuals with protrusive mandible tend to have higher composition of type II fibers. In this study, variations in correlation between the chromatin modifying enzymes and fiber type composition of occlusal subgroups were reported. Malocclusion has multifactorial etiology, and the degree of influence by muscle function is difficult to determine. Further study is needed to gain better understanding of the development of craniofacial structure and muscle fiber phenotype. Recent findings suggest that targeted delivery of microRNAs can potentially be used to specifically manipulate gene in clinical medicine. Development of drugs to target signaling pathways associated with myofiber remodeling may provide new approach for the correction of musculoskeletal disorders of craniofacial structures in the future.

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APPENDICES

APPENDIX A

LIMB DATA

HDAC4 RQ and MYST4 RQ Gene Expression of Limb Muscle

Muscle	HDAC4 RQ	MYST4 RQ	MYST4/ HDAC4
Forearm (Extensor+ Trapezius)	1.247	6.724	5.393
Triceps/Deltoid	1.251	6.690	5.348
Triceps	0.812	5.060	6.229
Quadriceps	1.234	5.729	4.644
Ave	1.136	6.051	5.404
n	4	4	4
StD	0.187	0.698	0.562
SEM	0.094	0.349	0.281

Percent MyHC Gene Expression of Limb Muscle

Muscle	I %	IIA %	IIIX %	IIA + IIIX(%)	Neo %	Atrial %	<u>N+A</u>
Forearm (Extensor+ Trapezius)	46.125	44.502	9.032	53.534	0.001	0.341	0.342
Triceps/Deltoid	59.845	29.456	10.207	39.663	0.001	0.491	0.492
Triceps	45.232	52.995	2.173	55.167	0.008	0.190	0.198
Quadriceps	51.630	30.686	16.995	47.682	0.405	0.284	0.689
Ave	50.708	39.410	9.607	49.011	0.104	0.326	0.430
n	4	4	4	4	4	4	4
StD	5.816	9.819	5.257	6.0727	0.174	0.109	0.182
SEM	2.908	4.910	2.629	3.0364	0.087	0.055	0.091

Fiber Type Percent Occupancy of Limb Muscle

Muscle	<u>Hybrid I/II</u>			
	<u>Type 1 Occ</u>	<u>Occ</u>	<u>Type IIA Occ</u>	<u>Type IIX Occ</u>
Forearm (Extensor+ Trapezius)	30.836	0	66.218	2.947
Triceps/Deltoid	47.969	0.0292	42.715	9.287
Triceps	39.629	0	49.975	10.395
Quadriceps	36.950	0.993	62.057	0
Ave	38.846	0.256	55.241	5.657
n	4	4	4	4
StD	6.156	0.426	9.375	4.330
SEM	3.078	0.213	4.688	2.165

APPENDIX B

HDAC4 RQ AND MYST4 RQ GENE EXPRESSION OF MASSETER MUSCLE

Sample	Sex	Age	Vertical	Sagittal	HDAC4	MYST4	MYST/HDAC
					RQ	RQ	
1	F	12	N	1	0.896	5.678	6.335
2	F	25	N	2	0.619	2.887	4.664
3	M	16	N	2	0.283	0.792	2.805
4	M	29	N	2	1.260	3.070	2.437
5	M	16	N	2	0.376	3.423	9.112
6	F	15	N	2	1.117	2.129	1.906
7	F	18	N	2	0.608	5.666	9.322
8	F	13	N	2	0.547	3.282	6.006
9	F	33	O	2	0.530	2.655	5.013
10	F	21	O	2	0.513	2.830	5.514
11	F	22	O	2	1.613	5.827	3.611
12	F	20	O	2	1.005	3.885	3.868
13	F	18	O	2	1.474	3.612	2.451
14	F	19	O	2	0.707	3.548	5.016
15	F	18	O	2	0.504	4.119	8.168
16	M	17	O	2	0.563	2.696	4.793
17	F	24	O	2	0.789	3.852	4.881
18	M	19	O	2	0.293	3.060	10.434
19	M	14	O	2	0.250	0.897	3.595
20	F	17	O	2	1.260	2.417	1.918
21	M	16	D	2	0.868	3.892	4.482
22	M	20	D	2	0.944	2.562	2.715
23	M	30	D	2	1.010	2.360	2.338
24	F	31	D	2	0.415	3.587	8.648

Sample	Sex	Age	Vertcal	Sagittal	HDAC4 MYST4		MYST/HDAC
					RQ	RQ	
25	M	18	D	2	1.909	4.616	2.417
26	F	18	D	2	1.229	2.741	2.230
27	F	15	N	3	0.958	5.824	6.077
28	F	15	N	3	0.972	4.251	4.373
29	M	17	N	3	0.497	3.148	6.332
30	M	20	N	3	3.335	4.986	1.495
31	F	17	N	3	0.245	3.195	13.057
32	M	18	O	3	1.138	5.674	4.985
33	F	16	O	3	2.284	4.414	1.932
34	F	32	O	3	1.095	2.369	2.164
35	M	32	O	3	1.371	6.482	4.727
36	M	15	O	3	1.105	6.822	6.176
37	F	34	O	3	5.864	5.579	0.952
38	F	15	O	3	0.865	2.630	3.039
39	M	19	D	3	1.162	3.872	3.331
40	M	27	D	3	0.532	2.651	4.985
41	F	17	D	3	1.620	6.793	4.193
42	M	22	D	3	1.050	4.736	4.511
43	M	17	D	3	0.456	2.508	5.498

APPENDIX C

FIBER TYPE PERCENT OCCUPANCY OF MASSETER MUSCLE

Sample	Age	Vertical	Sagittal	Type I Occ	Hybrid I/II Occ	Type II Occ	Type NeoA Occ
1	12	N	1	43.671	43.671	43.671	43.671
2	25	N	2	55.292	30.706	10.917	3.084
3	16	N	2	82.049	14.668	0.330	2.952
4	29	N	2	26.005	23.264	0	50.731
5	16	N	2	43.5	26.1	3.5	27.008
6	15	N	2	41.867	54.870	0.538	2.725
7	18	N	2	29.803	45.941	5.319	18.937
8	13	N	2	52.815	41.476	1.614	4.095
9	33	O	2	—	—	—	—
10	21	O	2	74.041	24.781	0.155	1.023
11	22	O	2	65.419	6.456	20.199	7.926
12	20	O	2	79.982	17.818	2.157	0.042
13	18	O	2	52.485	23.647	0.332	23.536
14	19	O	2	42.666	43.921	3.190	10.223
15	18	O	2	63.780	29.276	0.824	6.120
16	17	O	2	79.203	12.169	2.387	6.241
17	24	O	2	49.215	48.412	1.800	0.573
18	19	O	2	61.308	34.208	0.562	3.922
19	14	O	2				
20	17	O	2	36.434	58.873	2.470	2.224
21	16	D	2	42.236	16.211	34.142	7.411
22	20	D	2	39.108	40.276	10.953	9.663
23	30	D	2	45.547	4.673	37.264	12.516
24	31	D	2	36.919	45.493	13.721	3.867

Sample	Age	Vertical	Sagittal	Type 1 Occ	Hybrid I/II Occ	Type II Occ	Type NeoA Occ
25	18	D	2	—	—	—	—
26	18	D	2	66.785	1.860	31.355	0
27	15	N	3				
28	15	N	3	36.873	38.087	7.814	17.226
29	17	N	3	57.047	18.304	11.318	13.331
30	20	N	3	24.337	58.355	9.112	8.197
31	17	N	3	64.387	27.814	4.678	3.122
32	18	O	3	69.347	21.217	0	9.436
33	16	O	3	46.160	39.198	0.545	14.096
34	32	O	3	59.887	13.072	4.896	22.145
35	32	O	3	27.346	68.257	3.809	0.588
36	15	O	3	50.117	26.867	0.073	22.942
37	34	O	3	28.180	1.926	69.895	0
38	15	O	3	31.053	44.216	0.451	24.280
39	15	O	3	31.053	44.216	0.451	24.280
40	27	D	3	50.879	5.210	40.310	3.601
41	17	D	3	18.072	14.566	60.340	7.021
42	22	D	3	34.271	48.487	17.242	0
43	17	D	3	25.252	47.732	21.235	5.781

APPENDIX D

PERCENT GENE EXPRESSION OF MYHC DATA IN MASSETER MUSCLE

Sample	Sex	Age	Vertical	Sagittal	MyHC Data						
					I %	IIa %	IIx %	IIa + IIx (%)	Neo %	Atrial %	N+A %
1	F	12	N	1	43.671	43.671	43.671	43.671	43.671	43.671	43.671
2	F	25	N	2	46.764	4.833	16.393	21.226	13.563	18.446	32.010
3	M	16	N	2	82.44	0.77	1.13	1.89	3.26	12.42	15.68
4	M	29	N	2	19.547	9.169	31.404	40.573	8.471	31.409	39.880
5	M	16	N	2							
6	F	15	N	2	43.193	24.753	8.303	33.056	7.426	16.324	23.751
7	F	18	N	2	21.621	19.122	2.474	21.596	8.386	48.397	56.783
8	F	13	N	2	72.635	0.480	1.403	1.883	16.907	8.575	25.482
9	F	33	O	2	—	—	—	—	—	—	—
10	F	21	O	2	68.84	17.43	2.31	19.74	1.64	9.78	11.42
11	F	22	O	2	44.966	9.555	34.077	43.631	7.040	4.363	11.403
12	F	20	O	2	81.220	11.098	0.213	11.311	0.244	7.225	7.469
13	F	18	O	2	60.46	1.6	5.96	7.56	11.24	20.74	31.98
14	F	19	O	2	52.8	2.75	9.39	12.14	21.18	13.88	35.06
15	F	18	O	2	82.82	0.69	1.84	2.53	8	6.66	14.66
16	M	17	O	2	55.325	10.312	10.867	21.179	12.852	10.645	23.496
17	F	24	O	2	68.981	9.711	3.051	12.762	4.323	13.933	18.256
18	M	19	O	2	71.356	4.668	0.638	5.306	13.384	9.953	23.338

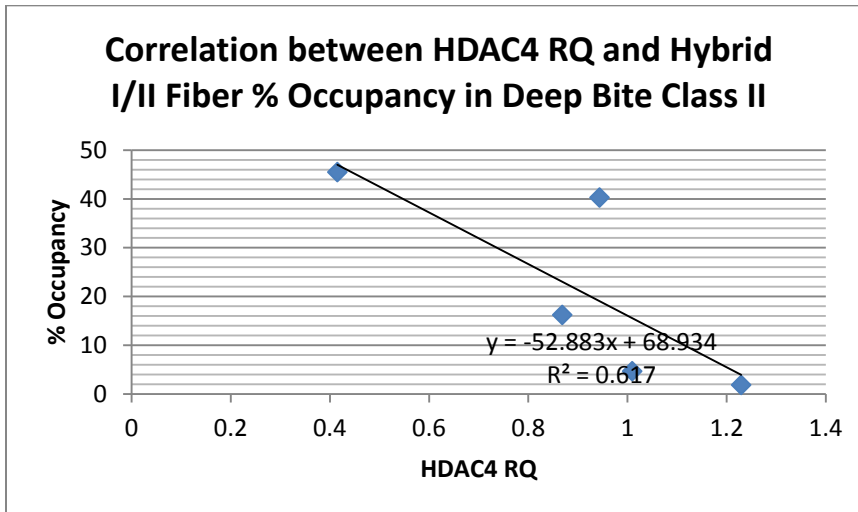
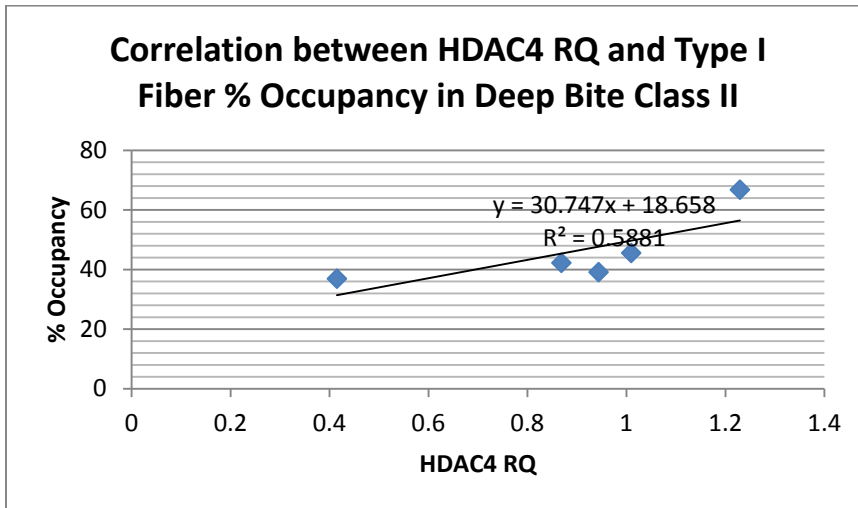
Sample	Sex	Age	Vertical	Sagittal	IIa + IIx (%)						
					I %	IIa %	IIx %	Neo %	Atrial %	N+A %	
19	M	14	O	2	60.595	36.971	1.828	38.799	0.250	0.356	0.606
20	F	17	O	2	27.154	53.795	3.849	57.643	5.233	9.968	15.201
21	M	16	D	2	32.969	6.463	38.036	44.499	4.842	17.690	22.532
22	M	20	D	2	62.647	1.420	2.761	4.181	8.431	24.741	33.172
23	M	30	D	2	36.2	6.85	45.64	52.49	0.41	10.9	11.31
24	F	31	D	2	46.483	9.412	3.515	12.926	5.487	35.103	40.590
25	M	18	D	2	56.996	5.238	2.819	8.057	21.980	12.966	34.947
26	F	18	D	2	57.374	20.297	11.491	31.787	1.631	9.207	10.838
27	F	15	N	3	59.475	26.746	3.437	30.183	6.032	4.310	10.342
28	F	15	N	3	54.058	4.721	2.966	7.687	8.389	29.866	38.255
29	M	17	N	3	59.436	0.738	14.070	14.807	4.534	21.223	25.756
30	M	20	N	3	64.515	1.974	12.690	14.664	18.643	2.177	20.820
31	F	17	N	3	82.505	8.507	1.987	10.494	3.085	3.917	7.001
32	M	18	O	3	63.776	5.249	5.798	11.046	7.955	17.222	25.178
33	F	16	O	3	68.186	16.365	2.944	19.10	4.999	7.5051	12.504
34	F	32	O	3	50.025	15.165	18.495	33.66	1.87	14.435	16.305
35	M	32	O	3	77.274	8.501	1.973	10.474	11.035	1.216	12.251
36	M	15	O	3	9.668	2.456	0.107	2.564	16.622	71.146	87.768
37	F	34	O	3	39.735	54.325	5.295	59.62	0.0015	0.645	0.6465
38	F	15	O	3	—	—	—	—	—	—	—
39	M	19	D	3	24.375	11.858	47.903	59.761	2.245	13.619	15.864

Sample	Sex	Age	Vertical	Sagittal	I %	IIa %	IIx %	IIa +	Neo %	Atrial %	N+A %
								IIx (%)			
40	M	27	D	3	48.43	11.58	33.91	45.49	1.82	4.26	6.08
								74.559	0.1038	8.3331	8.4370
41	F	17	D	3	17.004	65.044	9.515	13	36	94	3
42	M	22	D	3	—	—	—	—	—	—	—
								28.356	2.5733	12.624	15.198
43	M	17	D	3	56.445	20.802	7.554	65	93	65	04

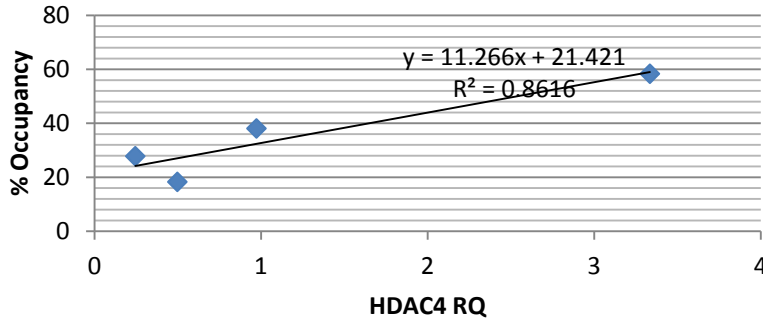
APPENDIX E

PLOTS OF HIGHLY CORRELATED VALUES BETWEEN THE CHROMATIN MODIFYING ENZYME AND PERCENT OCCUPANCY

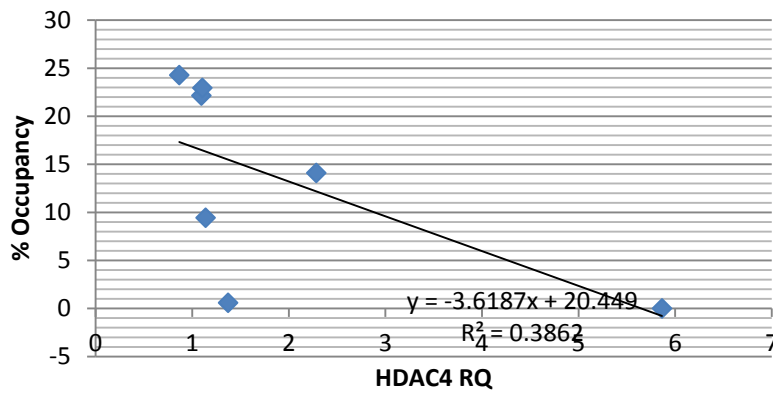
* R Values May Not Coincide with the Correlation Value Measured from Kendall's Tau-b Analysis as These Graphs Are Generated from Microsoft Excel Program



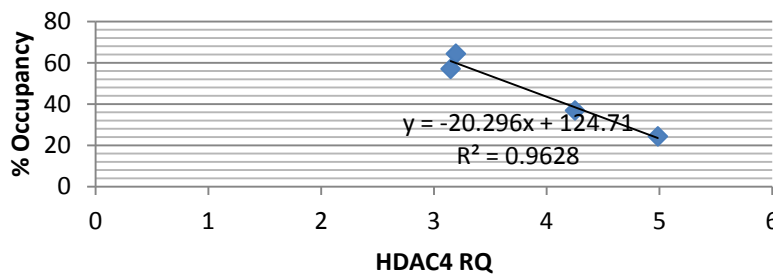
Correlation between HDAC4 RQ and Hybrid I/II Fiber % Occupancy in Normal Bite Class III



Correlation between HDAC4 RQ and N/A Fiber % Occupancy in Open Bite Class III



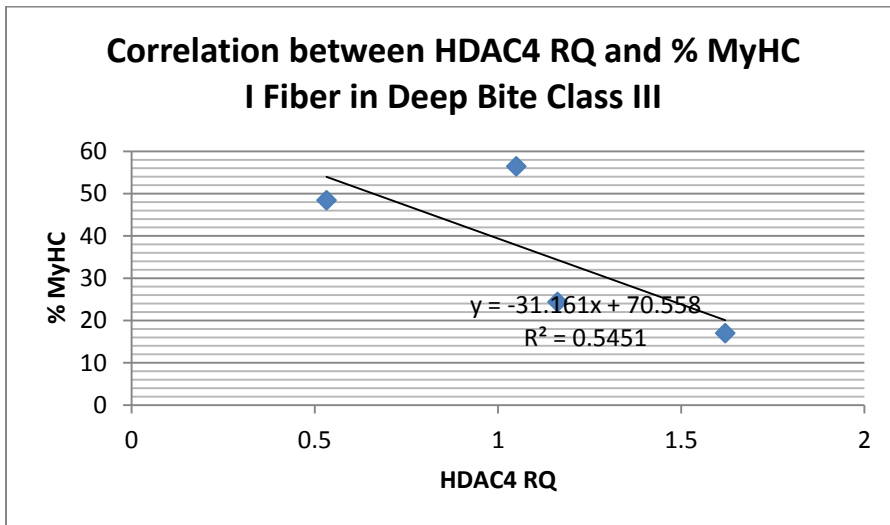
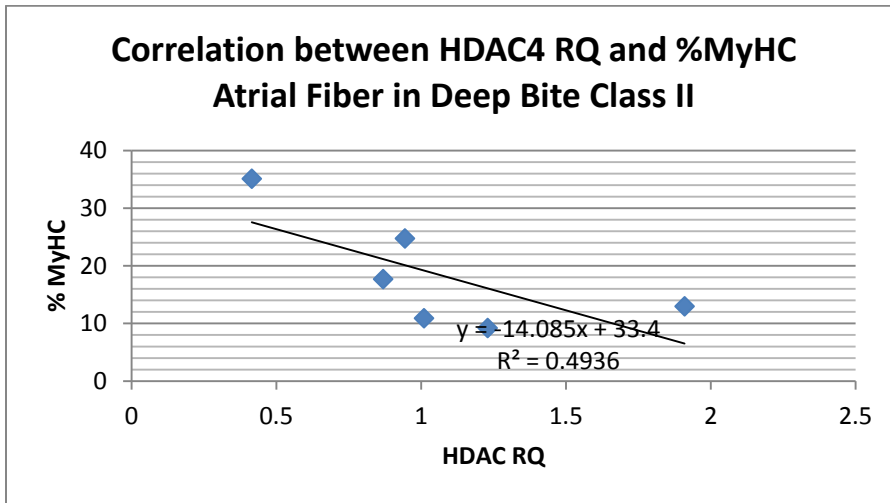
Correlation between MYST4 RQ and Type I Fiber % Occupancy in Normal Bite Class III

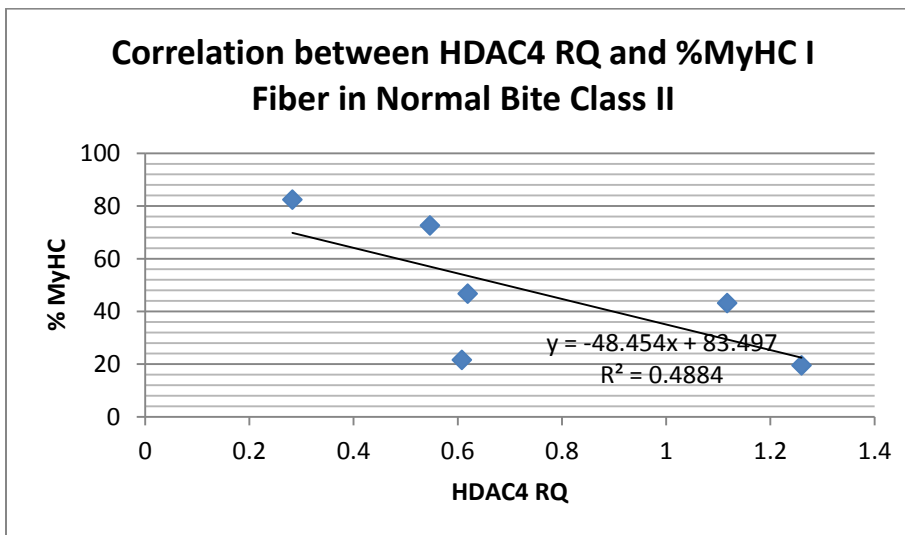
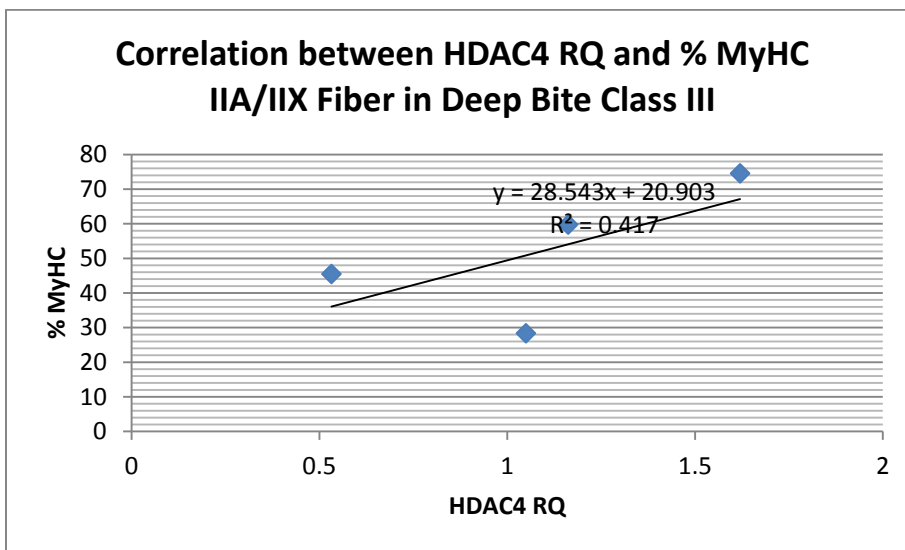
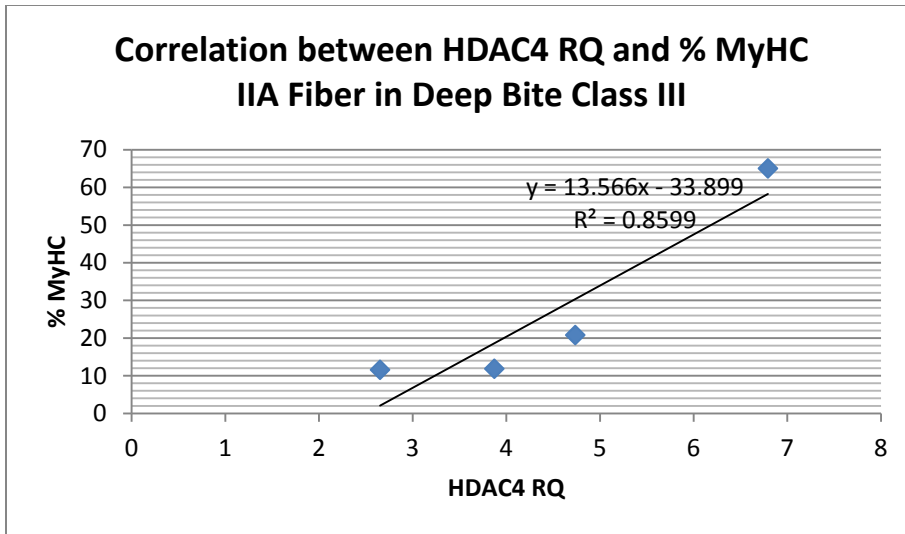


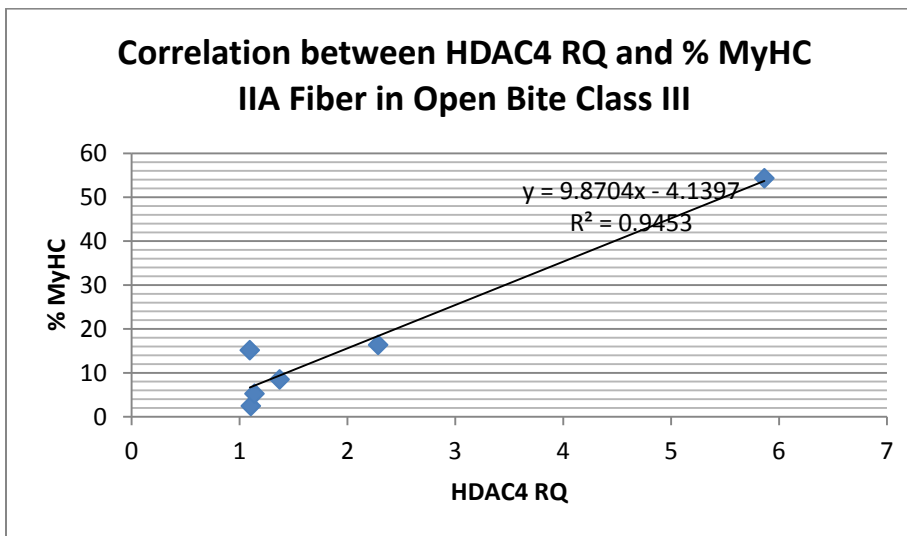
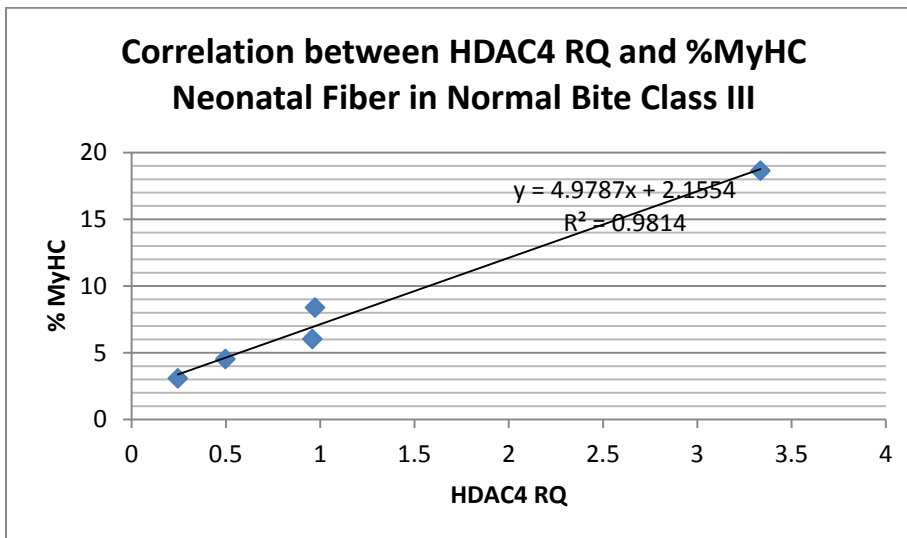
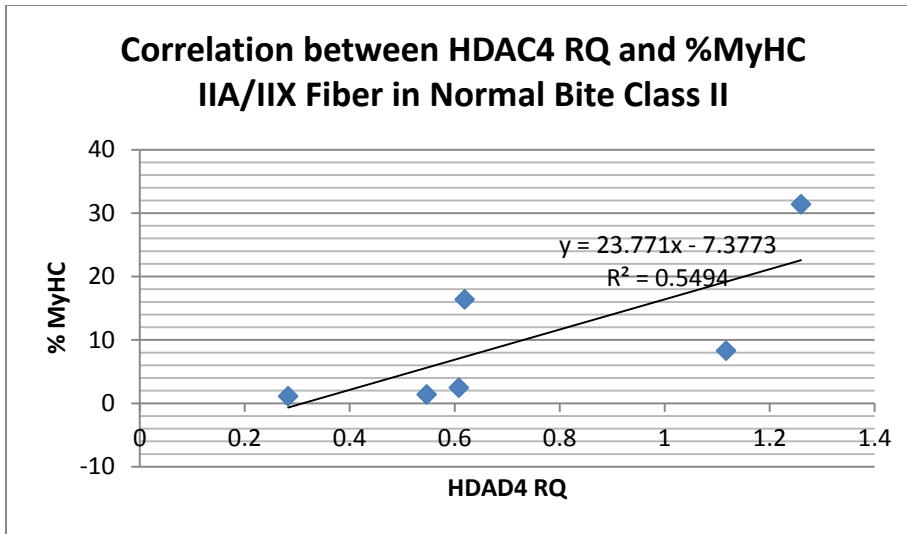
APPENDIX F

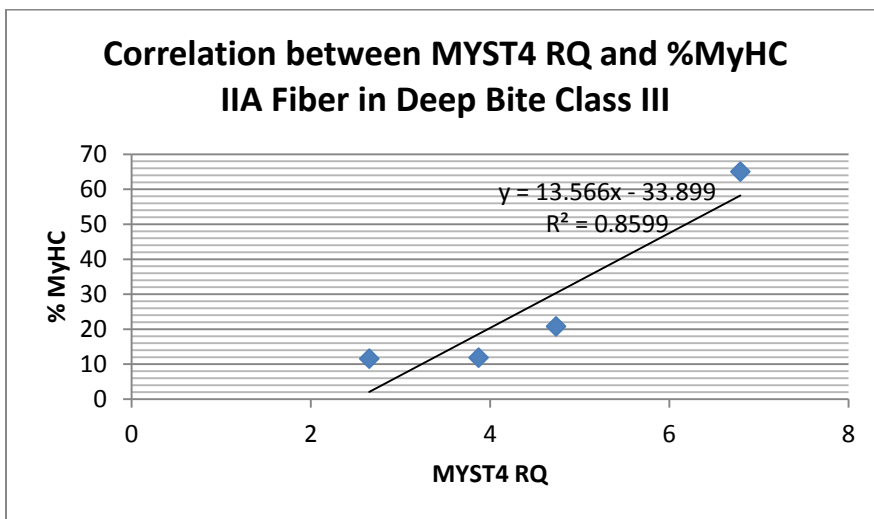
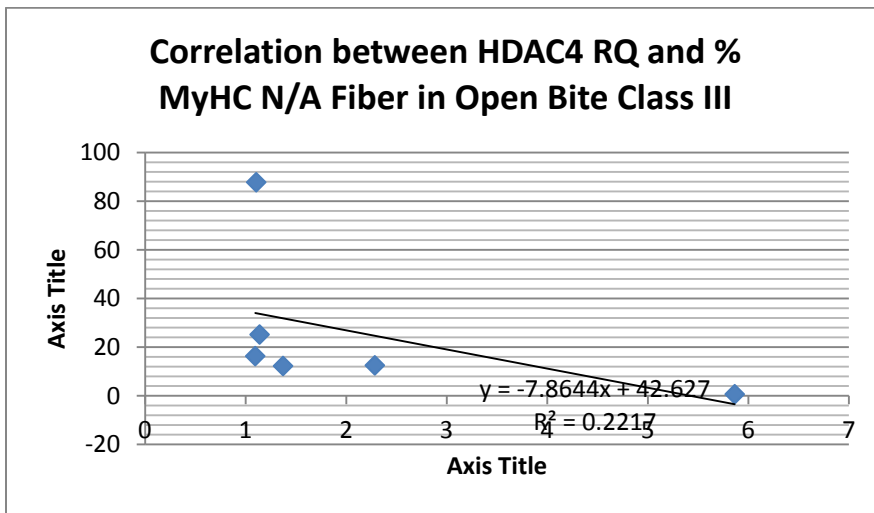
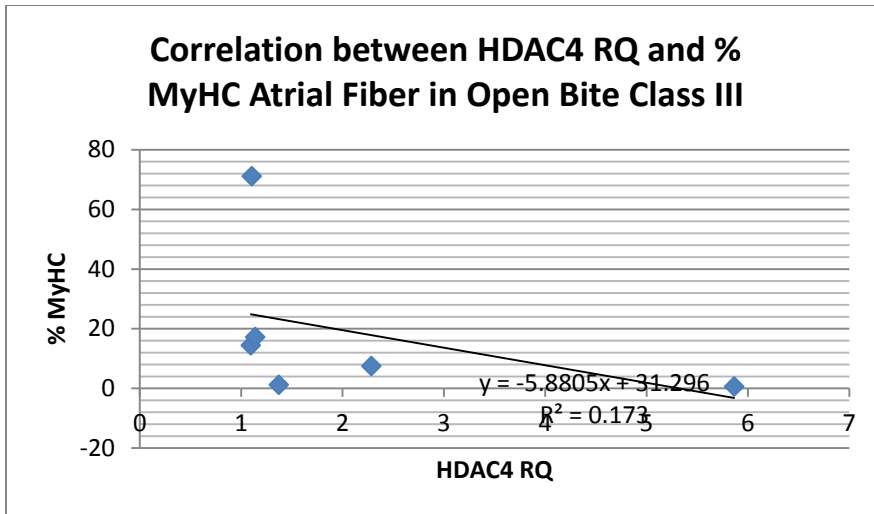
PLOTS OF HIGHLY CORRELATED VALUES BETWEEN THE CHROMATIN MODIFYING ENZYME AND PERCENT MYHC LEVEL

* R Values May Not Coincide with the Correlation Value Measured from Kendall's Tau-b Analysis as These Graphs Are Generated from Microsoft Excel Program)









APPENDIX G

GENERALIZED LINEAR MODELS

Generalized Linear Model of HDAC4 RQ and % Occupancy based on Gender, Age, Vertical Dimension and Sagittal Dimension

		Coef.	SE	Z	P	Lower 95% Ci	Upper 95% CI
Gender		-0.289	0.302	-0.930	0.352	-0.871	0.310
Age		0.044	0.027	1.640	0.100	-0.008	0.096
Vertical	2	-0.321	0.480	-0.670	0.504	-1.262	0.621
	3	-0.228	0.453	-0.500	0.615	-1.117	0.661
Sagittal		-0.119	0.467	-0.250	0.799	-1.035	0.797
Vert * Sag.	2 * 3	0.784	0.723	1.080	0.278	-0.633	2.202
	3 * 3	1.112	0.643	1.730	0.084	-0.148	2.372
Intercept		0.585	0.811	0.720	0.471	-1.006	2.175

Generalized Linear Model of HDAC4 RQ and % Occupancy based on Gender, Age and Six Subgroups

	Coef.	SE	Z	P	Lower 95% Ci	Upper 95% CI
Gender	-0.311	0.324	-0.960	0.337	-0.947	0.325
Age	0.052	0.029	1.820	0.069	-0.004	0.109
D3	0.269	0.590	0.460	0.648	-0.887	1.426
N2	-0.042	0.554	-0.080	0.939	-1.129	1.044
N3	0.639	0.639	1.000	0.317	-0.614	1.893
O2	0.038	0.530	0.070	0.943	-1.000	1.076
O3	1.006	0.541	1.860	0.063	-0.054	2.067
Intercept	0.185	0.937	0.200	0.844	-1.652	2.022

Generalized Linear Model of MYST4 RQ and % Occupancy based on Gender, Age, Vertical Dimension and Sagittal Dimension

		Coef.	SE	Z	P	Lower 95% Ci	Upper 95% CI
Gender		-0.102	0.459	-0.220	0.824	-1.003	0.798
Age		-0.003	0.041	-0.060	0.950	-0.082	0.077
Vertical	2	-0.200	0.732	-0.270	0.784	-1.635	1.234
	3	0.327	0.691	0.470	0.636	-1.027	1.681
Sagittal		0.622	0.712	0.870	0.382	-0.774	2.018
Vert * Sag.	2 * 3	0.240	1.102	0.220	0.827	-1.920	2.400
	3 * 3	0.679	0.979	0.690	0.488	-1.241	2.598
Intercept		3.430	1.236	2.770	0.006	1.007	5.853

Generalized Linear Model of MYST4 RQ and % Occupancy based on Gender, Age and Six Subgroups

	Coef.	SE	Z	P	Lower 95% Ci	Upper 95% CI
Gender	-0.177	0.485	-0.370	0.715	-1.129	0.774
Age	0.003	0.043	0.080	0.938	-0.081	0.088
D3	1.127	0.883	1.280	0.201	-0.602	2.857
N2	-0.010	0.829	-0.010	0.991	-1.635	1.616
N3	0.868	0.957	0.910	0.364	-1.007	2.743
O2	0.497	0.793	0.630	0.531	-1.057	2.050
O3	1.793	0.810	1.810	0.057	0.206	3.380
Intercept	3.236	1.402	2.310	0.021	0.487	5.984

Generalized Linear Model of MYST4 RQ and Percent MyHC based on Gender, Age, Vertical Dimension and Sagittal Dimension

		Coef.	SE	Z	P	Lower 95% Ci	Upper 95% CI
Gender		-0.024	0.044	-0.550	0.588	-0.114	0.066
Age		-0.571	0.477	-1.200	0.241	-1.546	0.403
Vertical	2	-0.581	0.819	-0.710	0.484	-2.253	1.092
	3	-0.254	0.737	-0.350	0.732	-1.759	1.250
Sagittal		1.245	0.887	1.400	0.171	-0.566	3.056
Vert * Sag.	2 * 3	0.041	1.216	0.030	0.973	-2.442	2.525
	3 * 3	0.901	1.164	0.770	0.445	-1.477	3.278
Intercept		4.209	1.190	3.540	0.001	1.780	6.639

Generalized Linear Model of MYST4 RQ and Percent MyHC based on Gender, Age and Six Subgroups

	Coef.	SE	Z	P	Lower 95% Ci	Upper 95% CI
Gender	0.039	0.030	1.290	0.208	-0.023	0.100
Age	-0.298	0.324	-0.920	0.366	-0.960	0.365
D3	0.089	0.603	0.150	0.884	-1.143	1.320
N2	-0.314	0.557	-0.560	0.577	-1.450	0.823
N3	0.267	0.595	0.450	0.657	-0.948	1.482
O2	-0.242	0.501	-0.480	0.632	-1.265	0.781
O3	0.941	0.545	1.730	0.095	-0.172	2.053
Intercept	0.405	0.809	0.500	0.620	-1.246	2.057

APPENDIX H
ANOVA MODELS

ANOVA Model on HDAC4 RQ and Percent Occupancy

Source	DF	SS	MS	F	P
Model	5	7.304	1.460	1.62	0.184
Error	32	28.937	0.904		
Corrected Total	37	36.242			

ANOVA Model on MYST4 RQ and Percent Occupancy

Source	DF	SS	MS	F	P
Model	5	15.699	3.139	1.76	0.149
Error	32	57.031	1.782		
Corrected Total	37	72.730			

ANOVA Model on HDAC4 RQ and Percent MyHC

Source	DF	SS	MS	F	P
Model	5	8.218	1.643	1.85	0.130
Error	32	28.390	0.887		
Corrected Total	37	36.609			

ANOVA Model on MYST4 RQ Percent MyHC

Source	DF	SS	MS	F	P
Model	5	23.317	4.663	2.510	0.060
Error	32	59.518	1.859		
Corrected Total	37	82.835			