

***DIFFERENTIAL EXPRESSION OF miRNA 328 IN MASSETER MUSCLES OF
SUBJECTS WITH FACIAL ASYMMETRY***

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ABSTRACT

MicroRNAs regulate posttranscriptional expression of target genes leading to inhibition or degradation of mRNA therefore inhibiting synthesis of protein coding genes. We reported that miRNA genes associated with response to ion-channel/transporter functions are differentially expressed in masseter of subjects with facial asymmetry. We have selected one species, *miRNA 328*, to test whether its expression associates with malocclusion types, asymmetry and TMD among orthognathic surgery patients.

RNA was isolated from muscle of patients undergoing mandibular sagittal-split surgical procedures for non-syndromic skeletal discrepancies. Subjects were diagnosed with one of the following types of malocclusion, with or without TMD and facial asymmetry: Class-II or Class-III with vertical normal, open or deep bite. A two-step process was used for quantification of miRNAs. RNA was reverse transcribed and assayed using specific TaqManTM primers and probe for *microRNA 328*. Standard curve experiments were used for assay analysis.

MicroRNA 328 expression values were low, ranging from 1-80pg throughout. Quantifiable differences were detected in comparisons between parameter groups. Statistical significant associations ($P < 0.04$) were found between female Class III and male Class III malocclusion subjects. In addition, interesting differences were identified among TMD-related myalgia groups with $p < 0.20$, and between male Class II and male Class III malocclusion subjects. Power analyses were done to determine the sample sizes needed for each group to attain significance.

MicroRNA 328 is differentially expressed in masseter of subjects with Class-II/Class-III malocclusion and in TMD-related myalgia. Larger sample sizes are needed to verify whether *microRNA 328* expression acts in both disorders. By power analysis, to reach $p < 0.05$ with a power = 0.8, 60 samples are needed in both TMD and Non-TMD groups. Differential expression of *microRNA 328* seen in Class III females and males suggests a potential influence on muscle target genes, craniofacial development and pain.

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CHAPTER 1

INTRODUCTION

Approximately 20 % of the human population worldwide presents with some type of distortion in jaw growth (Wolford & Fields, 1999). These non-syndromic distortions can produce severe malocclusion of the dentition requiring orthognathic surgery in addition to orthodontic treatment to correct the skeletal discrepancy (Proffit, 1998). In the United States, approximately 1.8 million people have severe dentofacial deformities in need of combined orthodontic and surgical treatment (Bailey et al., 1999).

The etiology of these malocclusions is complex and not fully understood. A variable number and combination of factors, including genetic, behavioral and environmental components, influence it. The soft tissue envelope and the muscles of mastication, through force application, are key environmental influences on the growth of the craniofacial structures, and can also have an effect on the long-term stability of orthognathic surgery.

Masticatory muscle function can affect craniofacial growth and contribute to variations in facial growth in the vertical dimension (Sciote, 2012). According to Sassouni, the vertical alignment of the jaw closing muscles directs skeletal growth towards a deep bite type of facial vertical deformity, whereas obliquely aligned jaw closing muscles lead to an open bite vertical deformity. Others have shown that fiber type composition between human limb and masseter muscles is very different (Rowlerson et al., 2005) and that fiber types properties are closely associated with variations in the

growth of the face (Sciote et al., 2012).

Malocclusion as the result of abnormal growth of the jaws can lead to disproportions of the craniofacial structures causing facial asymmetry and temporomandibular joint disorders (TMD). TMD is the most common chronic oro-facial pain condition with a higher frequency in woman than men (Maixner et al., 2011). The prevalence in women is approximately 25% in symmetric malocclusions and 32% in facial asymmetry. The etiology of TMD is multifactorial, but most are muscular in origin. Associations have been found between TMD and facial asymmetry as well as TMD and inflammatory/pain related genes.

A combination of transcription and growth factors acting on bone, teeth, and skeletal muscle influence malocclusions (Sciote, 2013). As complex traits, malocclusions can be the result of the interaction of genetic and epigenetic factors. With a range of 20,000-25,000 protein-coding genes and a number of known non-coding RNAs, such as transfer RNAs, ribosomal RNAs, small nucleolar RNAs (snoRNAs) and microRNAs in the human genome, a broad spectrum of variation is observed. (International Human Genome Sequencing Consortium, 2004).

MicroRNAs are a class of small non-coding RNA of approximately 20-25 nucleotides in length, that post-transcriptionally regulate gene expression by binding to target sites in the 3' untranslated regions of mRNA (Bai et al., 2014). MicroRNAs act by either inhibition of translation of mRNA (incomplete binding) or the degradation of mRNA (complete binding) leading to the inhibition of protein

synthesis of normally coding genes. In some instances microRNAs have been reported to activate translation of mRNAs.

Accordingly, because of the evidence for their diverse functions, miRNAs are recognized to be an important class of biomolecules in control of cellular processes.

Recent studies have identified microRNAs as having a key role in adaptive responses of the cells to the environment and vice versa, microRNAs can be controlled by epigenetic factors. The two major epigenetic mechanisms that affect miRNA expression are: DNA methylation and histone modification (Bai, 2007). These epigenetic activities provide fundamental ways by which cells and tissues may respond to extracellular stimuli during development and growth.

Approximately 30% of the protein-coding genome is regulated by miRNAs, involving multiple physiological biologic processes including proliferation, differentiation, cell growth, apoptosis and metabolism (Bai et al., 2014). Since their discovery, numerous studies have associated miRNAs with the pathogenesis of diseases such as cancer, cardiovascular diseases, autoimmune diseases, inflammatory muscle pain, stem cell differentiation and phenotypic variations in muscle fibers. As a result, microRNAs can be useful molecular biomarkers in the diagnosis of disease and may represent potential therapeutic targets.

CHAPTER 2

REVIEW OF THE LITERATURE

2.1 Development of Malocclusion

Malocclusion affects the majority of today's population. But although the prevalence of malocclusion is considerably high in the modern world, it is a relatively new human disease and a "disease of civilization," signifying that it is more prevalent in developed, urbanized areas (Corruccini 1984). The trait appears within populations after a decrease in the functional demand for chewing, caused by the transition to softer processed foods, and can occur rapidly within 1 or 2 human generations. Anthropologic studies have observed the changes in occlusal relationships and shape of the jaws associated with a decreased functional demand for chewing. Westin Price, in his studies about the relationship between nutrition and physical degeneration, compared the dentition and facial form of residents of Australia, New Zealand, South America and other populations, to that of their ancestors. He found that the size and shape of the dental arches, the strength of the masseter muscles and the breadth of the face are different between the primitive and the modernized populations. Progressive degeneration of facial form with narrower dental arches, crowding, and elongated faces are seen on individuals exposed to modern diets.

Studies of the Inuit and Neanderthal skull have also exhibited a correlation between masticatory function and craniofacial form. Neanderthal facial configuration is thought to have adapted by an anterior migration of the masseter muscles and a decrease

in the distance between the TMJ and the incisors, to allow for a more efficient use of the anterior dentition. Similarly a more anterior position of the masseter and temporalis muscles of the Inuit was observed, along with molar bite forces 2 to 3 times higher than in any modern population. This is attributed to their dietary behavior, such as the consumption of hard meats and crunching of bones (Spencer et al., 1977).

Experimental animal studies support this theory and show a causal relationship between changes in dietary consistency and growth of the craniofacial skeleton. Animals raised on softer diets have significantly less bite force, altered fiber type composition and decreased muscle fiber size. In rodent studies, soft diet results in decreased weight, decreased condylar and mandibular growth, while hard diet results in increased weight, increased volume and thickness of bone. In growing rats, in which unilateral jaw muscles were exercised, changes in the cranium, dento-alveolar complex and occlusion of the ipsilateral side were observed (Horowitz, 1955). These changes are thought to be the result of epigenetic changes in gene expression of the connective tissue.

Strong evidence of the effect of muscle activity on craniofacial growth is also found in studies of individuals suffering from Duchenne muscular dystrophy, a progressive disease of muscle weakness in which the lack of activity of the masticatory muscles and the increased tonus of the perioral muscles result in sagittal under development.

Muscle weakening of the elevator muscles of the mandible leads to a clockwise rotation of the mandible, producing an open bite malocclusion and long facies. The

weakness of the masticatory muscles allows unrestrained growth in the transversal dimension. This transverse discrepancy is also stimulated by the position of the tongue (Eckardt, 1996).

On the contrary, increased muscular force and contraction of the muscles of mastication can result in a counterclockwise rotation of the mandible in children with long faces (Ingervall, 1987). If excessive, muscular contraction can restrict growth of the affected side of the body, as seen in patients affected with torticollis (Lee, 2012).

2.2 Masseter Muscle Fiber Types

The fiber type composition of the masseter muscles varies greatly from the composition of the majority of the skeletal muscles throughout the body. The fiber composition of the skeletal muscles of the limbs and abdomen is primarily composed of three main fiber types: type I, IIA, and IIX myosin heavy chain isoforms, whereas the masticatory muscles are characterized by 4 major fiber groups: type I fibers containing only type I myosin; type II containing type IIA and/or IIX myosin; type I-II hybrid fibers containing both isoforms I and II; and neonatal-atrial fibers containing a combination of type I, II and neonatal and /or α -cardiac myosin (Laakso, 2008) (Sciote, 1994).

These different isoforms have different functional demands and roles. Contraction velocity increases successively from type I, type IIA, type IIX, type IIB, while a decrease in fatigability follows that same order (Bottinelli *et al.*, 1996). In addition, there is a difference in the diameter of the fibers between the skeletal and jaw muscles, in skeletal muscles type II fibers are larger in diameter than type I fibers, while the opposite is seen

in jaw muscles. The smaller cross-sectional area is thought to facilitate oxygen exchange and improve muscle resistance to fatigue.

Type I fibers are present in large quantities in postural muscles. Due to their slow contracting rate and resistance to fatigue, Type I fibers have the lowest energy demand and are the most economic. In contrast, Type II fibers are fast contracting, high fatigue fibers, making them the least economic and most energy demanding fiber type. The Type II fibers can be subdivided into IIA with an intermediate energy cost and a fast shortening speed, and Type IIX the fiber type with the highest energy cost and fastest shortening speed (Sciote, 1994). Neonatal and atrial fibers can also be found as minor components and present an unusual motor protein expression, of which properties and roles are presently not fully understood.

The masseter muscle is unique in the high proportion of hybrid fibers present in comparison to limb muscles, ranging from two fiber isoforms to any possible combination (Rowlerson, 2005). The type I/II hybrids share characteristics of both type I and type II fibers, with intermediate capacity (Morris, 2001). A great variability in composition of the fibers within the same muscle in different individuals is seen. Genetic influences in conjunction with systemic variations and local influences are thought to be the cause of this. Fiber-type composition has also been associated with age and gender, fiber activation- and stretch, behavioral differences such as bruxism, the use of dental prostheses, etc.

Vertical dimension malocclusions have been shown to have an association with some fiber types when these fibers are present in masseter muscle. (Rowlerson, 2005).

Predominance in occupancy of type I muscle fibers is seen in open bites, type II fiber occupancy in deep bites and predominance of type I along with type I/II hybrid type in class III subjects with deep bites (Sciote 2005)(Fig.1). Notably, the predominance of type II fibers is seen in cases of facial asymmetry on the side of the deviation or “short side”. In contrast, no significant fiber type size or occupancy differences are found in symmetric individuals (Sciote, 2013).

Gender may also play a role, but sagittal facial dimension has no relation to the fiber type composition. In masseter muscle of young male adults, studies have found a larger number of type II fibers, while females had a larger number of type I and IM fibers (Korfage et al., 2005).

Figure 1.

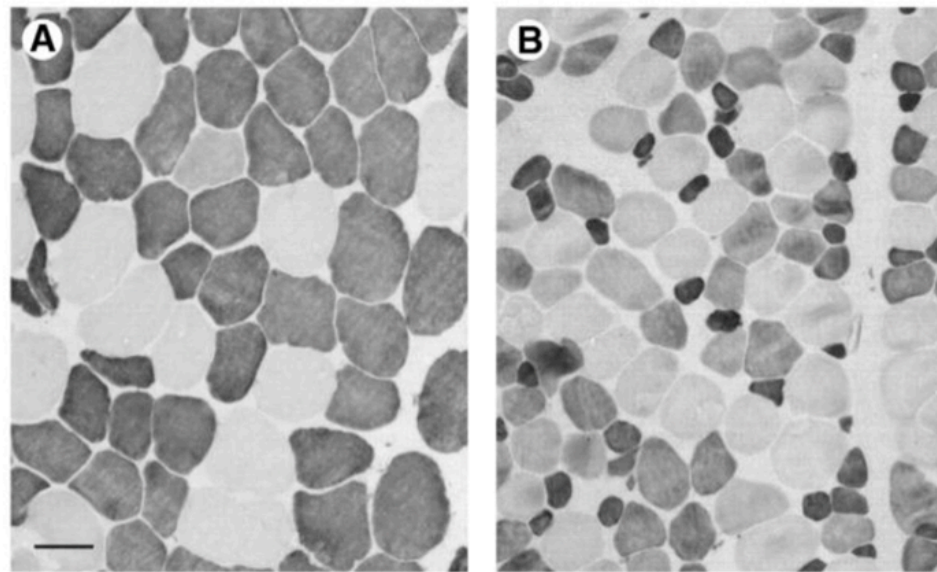


Figure 1. Mean differences in type I (pale stained) and type II (strongly stained) fiber areas in masseter muscle of **A.** Deep bite patient, compared with **B.** Normal bite patient. Adapted from Sciote et al. (2012) Human masseter, malocclusions and muscle growth factor expression. *Journal of Oral and Maxillofacial Surgery*, 70, 440–448.

2.3 Temporomandibular Disorders and Facial Asymmetry

Facial asymmetries as a result of abnormal jaw growth can lead to comorbid conditions such as temporomandibular joint disorders (TMD). TMD, is the most common chronic oro-facial pain condition. The principal symptom observed is masticatory muscle pain that can be referred to the head and neck area (Schiffman et al., 1990). Muscle dysfunction and limited mouth opening are also seen.

TMD is more commonly reported in women than males with a ratio of 2:1 in the general population (Maixner et al., 2011). The prevalence in women varies depending on the malocclusion with a prevalence of approximately 25% in symmetric malocclusions and 32% in facial asymmetry. The most reliable socio-demographic factors predictive of TMD are gender and age. TMD prevalence is higher in people 40 and older and decreases in people of both younger and older age groups. Race and ethnicity are not reliable demographic factors associated with prevalence of TMD pain.

Although mostly of muscular origin (Schiffman et al., 1990), multiple risk factors can contribute to the development of TMD. Muscular trauma, bone and connective tissue disorders, hormonal differences, depression and anxiety are known risk factors for the development of TMD. More recently, associations have been found between TMD and facial asymmetry (Fushima et al., 1999), and between TMD and inflammatory/pain related genes. Genetic variations affecting pain sensitivity coupled with environmental factors have been found to produce TMD vulnerable phenotypes (Maixner et al., 2011).

2.4 Epigenetic regulation of Pain

The epigenetic regulation, DNA-sequence-independent mechanisms that can regulate gene expression, underlying pain have been currently classified into three major molecular mechanisms: DNA methylation, chromatin remodeling and non-coding RNA.

DNA methylation is a complex event that occurs mostly on the CpG dinucleotide (carbon 5 of the pyrimidine ring of the cytosine residue followed by guanine residue). Methylated CpGs recruit several nuclear proteins known as CpG binding proteins and repel other transcription factors, causing the down regulation or silencing of gene transcription. DNA methyltransferase 1,3a,3b, are the enzymes responsible for catalyzing this process. Clusters of CpGs are distributed within small guanine-cytosine rich areas known as islands (CGIs), which usually appear near the transcription start sites of approximately 72%-76% of human protein coding genes.

Many of the genes involved in persistent pain have been found to regulate or be regulated by DNA methylation. Tajerian et al. 2011, studied the transcriptional downregulation of the extracellular matrix protein SPARC (Secreted Protein, Acidic, Rich in Cysteine) SPARC in the disc of aging mice and found that DNA methylation is related to the degeneration of the discs and lower back pain.

Another epigenetic regulatory mechanism of gene expression is chromatin remodeling. The dynamic process of chromatin modification allows access to genomic DNA around the histone core. Lysine and arginine residues in the histone core can be

acetylated by the enzyme acetyltransferase causing the release of DNA, and activating gene expression.

On the contrary acetylated histones can be deacetylated by histone deacetylases leading to the reestablishment of the nucleosome, and suppression of gene expression. Currently conflicting results have been reported regarding the changes in hypersensitivity by these two reactions.

Non-coding RNAs (ncRNAs) are small RNAs that do not encode protein, but regulate gene expression at the transcription or translational levels. Among the most studied ncRNAs are miRNAs. These small non-coding molecules are involved in many developmental, physiological and pathophysiological processes altering protein expression, including pain perception and chronification (Niederberger et al., 2011).

2.5 MicroRNA History and Perspective

MicroRNAs are a class of small non-coding RNA of approximately 20-25 nucleotides in length, which can regulate expression of target genes post-transcriptionally by binding to target sites in the 3' untranslated regions of mRNA. MicroRNAs act by either inhibition of translation of mRNA or the degradation of mRNA leading to the inhibition of proteins synthesis of protein coding genes.

They are initially transcribed as primary transcripts (pri-miRNA). Within the nucleus, this primary structure is cleaved by the action of RNAse III endonuclease Drosha and the double stranded RNA binding protein DGCR8, to produce a 70-100 bp RNA molecule known as precursor microRNA (Kirby, 2013).

The pre-miRNA is then transported out of the nucleus where it is cleaved by Dicer, a second RNase III endonuclease, producing the mature microRNA (Fig. 2).

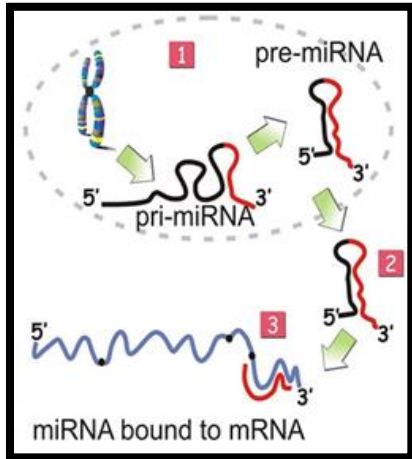


Figure 2. Schematic representation of mRNA molecule formation and binding to mRNA

MicroRNAs were first discovered in 1993 by Ambro's and Ruvkun's laboratories in the nematode *Caenorhabditis elegans*. The loss of function of *Lin-4*, the first microRNA discovered, causes failure of development of some larva structures. Seven years later in the year 2000, a second *miRNA Let-7* was discovered. This heterochronic gene of *C. elegans*, is conserved across species from flies to humans. The *Let-7* family is comprised of 12 miRNAs in humans. This discovery sparked an interest in the study of miRNAs, their possible human targets and functions (Fig. 2).

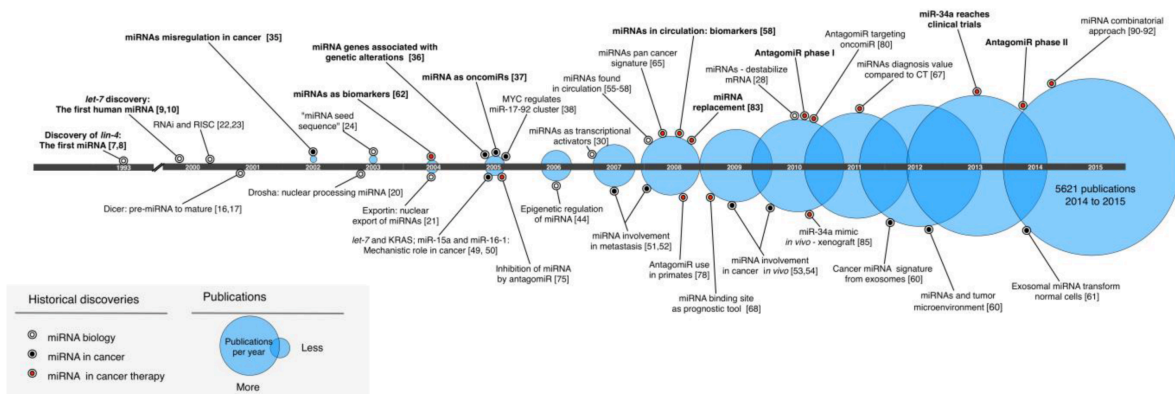


Figure 3. Historical discoveries and evolution of knowledge of microRNAs. Adapted from Orellana, E.A.; Kasinski, A.L. MicroRNAs in Cancer: A Historical Perspective on the Path from Discovery to Therapy. *Cancers* 2015, 7, 1388-1405.

In 2002 the first miRNAs thought to play an important role in the pathogenesis of chronic lymphocytic leukemia were reported (Calin et al., 2004). To date, alterations in miRNA expression have been reported in almost all cancer types, acting as either tumor suppressors or oncogenes. In mice studies, overexpression of *miR-21* lead to the development of a pre-B malignant lymphoid-like phenotype, demonstrating that *mir-21* is a genuine oncogene. When *miR-21* was inactivated, apoptosis caused the tumor to regress (Medina 2010).

MiRNA regulation is extremely sensitive to stimuli in the microenvironment; their response to these stimuli is considered one of the major mechanisms for epigenetic modification of the cell. Alterations in the expression and function of ion channels/transporters result in changes in miRNAs. Ion channels/transporters are expressed in almost all cell types and regulate the movement and distribution of ions

across the plasma membrane. Both the miRNAs and the ion channels/transporters are extremely sensitive to the cellular microenvironment, and ion transport mediate linking extracellular signals to intracellular miRNA alteration, which then influences a series of physiological and pathologic process. Stimuli such as hypoxia, variations in pH, temperature and ion concentrations can affect post-transcriptional miRNA-based regulation.

The notion that ion channels/transporters may be regulators of miRNAs by sensing changes in the microenvironment has led us to believe that miRNAs have the potential to react to almost all cellular mechanisms and act as key epigenetic regulators (Jiang, 2012).

2.5.1 *MicroRNA 328*

Skeletal muscle is able to increase in mass in response to mechanical loading, but it can also regenerate after damage via intrinsic regulation of gene transcription. Both cellular processes, hypertrophy and muscle regeneration, are mediated by the activation, proliferation and differentiation of satellite cells and appear to be modulated by the mitotic and myogenic activity of locally produced insulin-like growth factor 1 (IGF-1).

The signaling pathways of IGF-1 regulate critical biological processes such as aging and development. Dysregulation can lead to multiple neurodegenerative and metabolic disorders. This makes the IGF-1 signaling pathway an important possible target for the development of therapeutic and intervention strategies.

IGF-1, unlike other factors, acts as both a mitogen and a differentiation factor

during muscle development, regeneration, or hypertrophy. Cardiac hypertrophy has been inversely correlated with the expression of *miR-1* (Care et al., 2007) and *miR-1* depleted mice show cardiac defects, including misregulation of cardiac morphogenesis, electric conduction, and cell proliferation (Yang et al., 2007).

Recent studies have implicated *miR-328* in responses to various cellular processes. In mice studies, researchers have found that hypoxia can produce a significant inhibition of *miR-328* expression, and that *miR-328* inhibited L-type calcium channel- $\alpha 1C$ expression through a *miR-328* binding site within the 3' untranslated region of L-type calcium channel- $\alpha 1C$. Furthermore, *miR-328* has been shown to suppress the IGF-1 receptor, ultimately leading to apoptosis of pulmonary arterial smooth muscle cells. This has led to the conclusion that *miR-328* plays an important role in regulating multiple gene targets (Guo 2012).

CHAPTER 3

AIMS OF THE INVESTIGATION

The goal of the study that we propose here is to determine whether expression of miRNAs differed between malocclusion subjects with facial asymmetry from our microarray analysis.

The second phase, will be to investigate 3 specific microRNAs selected from the microarray of masseter muscle of subjects of the study population for malocclusion, facial asymmetry and temporomandibular joint disorder (TMD).

In a third phase, we will select the microRNA that demonstrates it is best for quantification by two-step RT-PCR methodology and identify associations between its expression and different genotypes, types of malocclusions, TMD and pain.

CHAPTER 4

MATERIALS & METHODS

4.1 Subjects

Sixty-two subjects (42 women and 20 men), with a mean age of 20 years old, participated in the study. The subjects presented with malocclusion requiring combined orthodontic and surgical treatment were recruited from the University of Lille Department of Oral and Maxillofacial Surgery. The individuals were of French/Caucasian descent, except for four of African descent and one of Parsee descent. Subjects signed an informed consent, and the French Independent Ethical Committee, Temple University, and the University of Pittsburgh IRB Committees validated the research protocol. Orthodontic diagnoses of these individuals were formulated by use of the Delaire's analysis (Delaire, 1981) and the jaw repositioning required executing the surgical treatment plan.

Subjects were classified into one of six groups based on vertical and sagittal dimensions: Class II normal bite, Class II deep bite, Class II open bite, Class III normal bite, Class III deep bite, Class III open bite. The diagnosis also includes the presence or absence of skeletal asymmetry. 32 of the 42 female subjects had Class II malocclusions (15 open bite, 9 deep bite, and 8 normal vertical occlusion) and 10 had Class III malocclusions (4 open bite, 5 deep bite and 1 normal vertical occlusion). Of the 20 male subjects, 11 had Class II malocclusions (2 open bite, 4 deep bite, and 5 normal vertical occlusion) and 9 had Class III malocclusions (3 open bite, 1 deep bite, and 5 normal

vertical occlusion) [Appendix A].

Masseter samples were 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 , after snap freezing, prior to histologic and gene expression analysis (Sciote, 2012; 2013). All surgical patients in this study completed the Jaw Pain and Function (JPF) questionnaire. The JPF questionnaire consists of eight questions regarding pain and an additional five questions regarding function (Gerstner, 1994; Undt, 2006) [Appendix B]. Eighteen of 62 subjects had JPF scores of ≥ 5 with signs of TMD required by the JPF questionnaire for diagnosis.

4.2 RNA isolation and Microarray

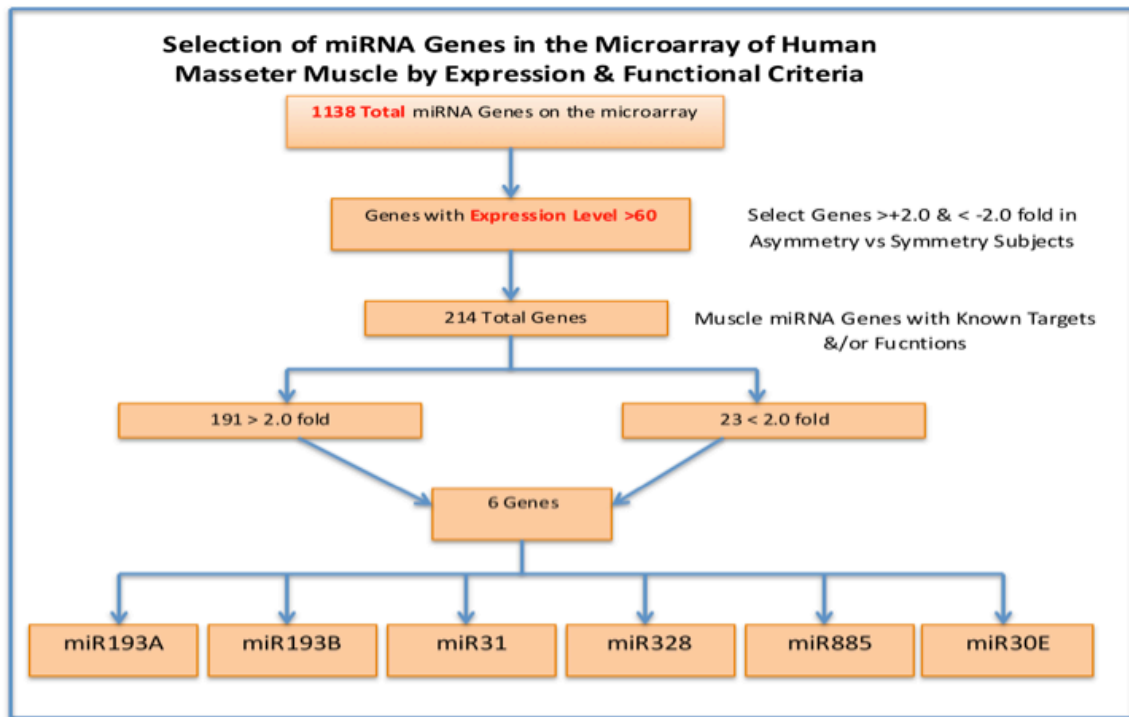
Samples of saliva were obtained, stored in Oragene® kits and used for DNA extraction and genotyping. A microarray analysis of reverse transcribed total RNA isolated from masseter biopsies from 11 selected subjects was performed at the University of Pennsylvania Center for Musculoskeletal Disorders. Total RNA was isolated using Qiagen miRNeasy® procedures (Qiagen Inc., Valencia, CA) according to the manufacturer's specifications. Control tests to assure the quality of the samples of isolated total RNA were performed by Agilent Bio analyzer® (Agilent, Santa Clara, CA) and Nano drop (Thermo Fisher, Waltham, MA) Nano drop spectrophotometry.

Muscle total RNA from 11 subjects, 2 of whom had posterior facial asymmetry, was analyzed on an Affymetrix HT2.0 microarray. Data from one subject with severe sleep apnea was excluded. A t-test was performed comparing the 2 asymmetric samples

to the 8 symmetric samples; resulting P values were corrected for false discovery rate because of relatively small sample sizes. Differences were considered significant if step-up $P < 0.02$ and fold differences were greater than ± 2 between groups.

Data from $>70,000$ transcripts were normalized and fold differences calculated using Partek Genomics software. The approximately 1,100 miRNAs represented on the array were summarized according to their expression intensities and fold differences between subjects with and without facial asymmetry (Table 1).

Table 1.



4.2.1 Protocol for Quantitative RT-PCR of micro-RNAs

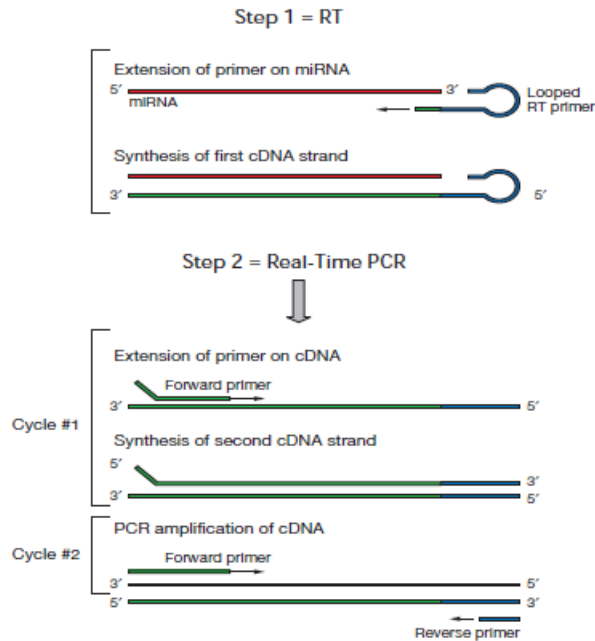


Figure 4. Two-step RT-PCR. From: Applied Biosystems TaqMan® MicroRNA Assay protocol.

Quantitation of miRNAs was done by a two-step process (Fig. 4). Reverse transcription of muscle RNA was first done as individual reactions for each miRNA of interest in the presence of a specific looped primer. In step 2, primed cDNAs were amplified by real time PCR reactions. Fig. 4 shows the TaqMan™ quantitation method using fluorescence-labeled primer-probe sets for each specific miRNA gene of interest.

4.2.2 Preparing the RT Reaction Master Mix and Reverse Transcription

A common stock of master mix was first prepared for all reactions of step 1 (Table 2). Proportionate amounts of primer for specific miRNA genes were added to separate aliquots of the master mix. Approximately 10ng of masseter muscle total RNA was added to the primer-supplemented master mix and reverse transcription of the individual miRNAs was done in a thermal cycler using conditions shown in Table 3.

Table 2. Master Mix for Reverse Transcription

Component	Master Mix Volume/15- μL Reaction
100mM dNTPs (with dTTP)	0.15
MultiScribe™ Reverse Transcriptase, 50 U/μL	1
10 X Reverse Transcription Buffer	1.5
RNase Inhibitor, 20U/iL	0.19
Nuclease-free water	4.16
Total	7

Table 3. Conditions for Reverse Transcription

Step Type	Time (min)	Temperature (°C)
Hold	30	16
Hold	30	42
Hold	5	85
Hold	∞	4

4.2.3 Preparing the PCR Reaction Master Mix and Quantification of miR-cDNAs

The master mix for quantitative PCR was prepared using the reagents and proportions shown in Table 4. For quantitative reactions, miRNA-specific primer-probe sets were added to the master mix along with an aliquot of the gene-matched miR-cDNA. Quantification was enabled by TaqMan™ fluorescence methods (Fig. 5).

Table 4. Components for Quantitative PCR

Component	Volume (μL) /50 μL Reaction
TaqMan MicroRNA Assay (20 \times) ; Primer-Probe	2.5
Product from RT reaction	6.66
TaqMan TM 2X AmpliTaq Gold	25
Nuclease-free water	15.8

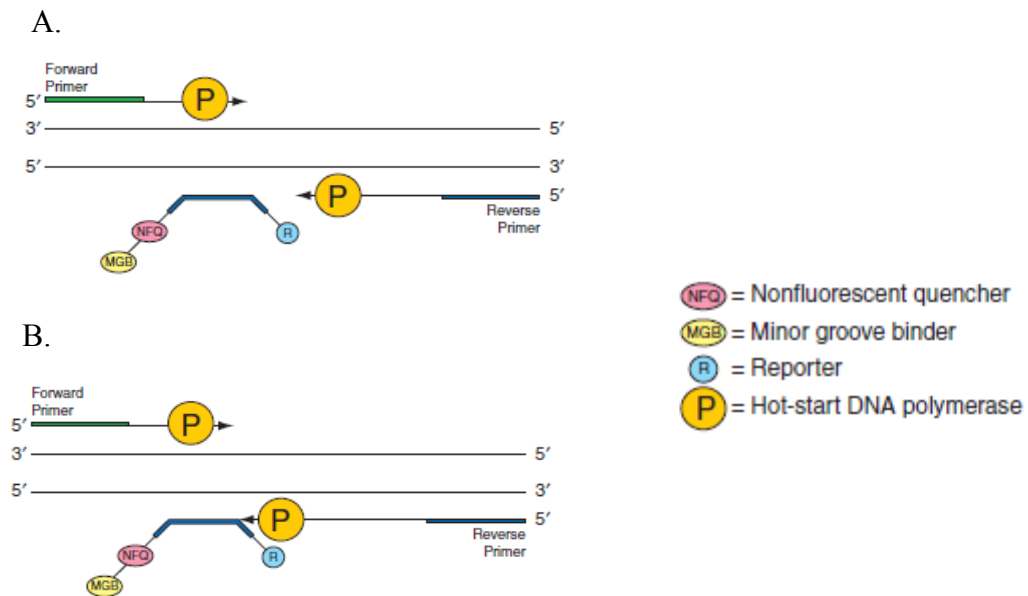


Figure 5. Methodological basis for quantitative PCR using TaqManTM primer – probe assays. A. Configuration of primers and fluorescent probe at the start of each cycle. B. DNA polymerase disengages probe allowing activation of reporter fluorescence during each cycle of amplification. From: Applied Biosystems Manual

Triplicate 15µl volumes from the 50µl reaction mix in Table 4 were assayed in 96 well reaction plates in an Applied Biosystems One-Step Fluorescence thermal cycler.

Amplification reactions were done according to conditions shown in Table 5.

Table 5. Parameters for quantitative PCR

Step	Enzyme Activation		
	Hold	PCR CYCLE (40 cycles)	
		Denature	Anneal/Extend
Time	10 min	15 sec	60 sec
Temp (°C)	95	95	60

4.2.4 Data Analyses

Fluorescence data from assays were analyzed by standard curve experiments. Standards were prepared from a pool of all masseter muscle RNA and used in each amplification experiment. RNA used as standards was reverse transcribed and amplified using looped RT-primers and primer probe assay sets for *microRNA 328* (Fig. 6). Properties for the standard curve of *microRNA 328* are summarized in Table 6. Based on the results of this experiment the $\Delta\Delta CT$ method could not be applied to quantify the expression of *microRNA 328* in the masseter muscle samples.

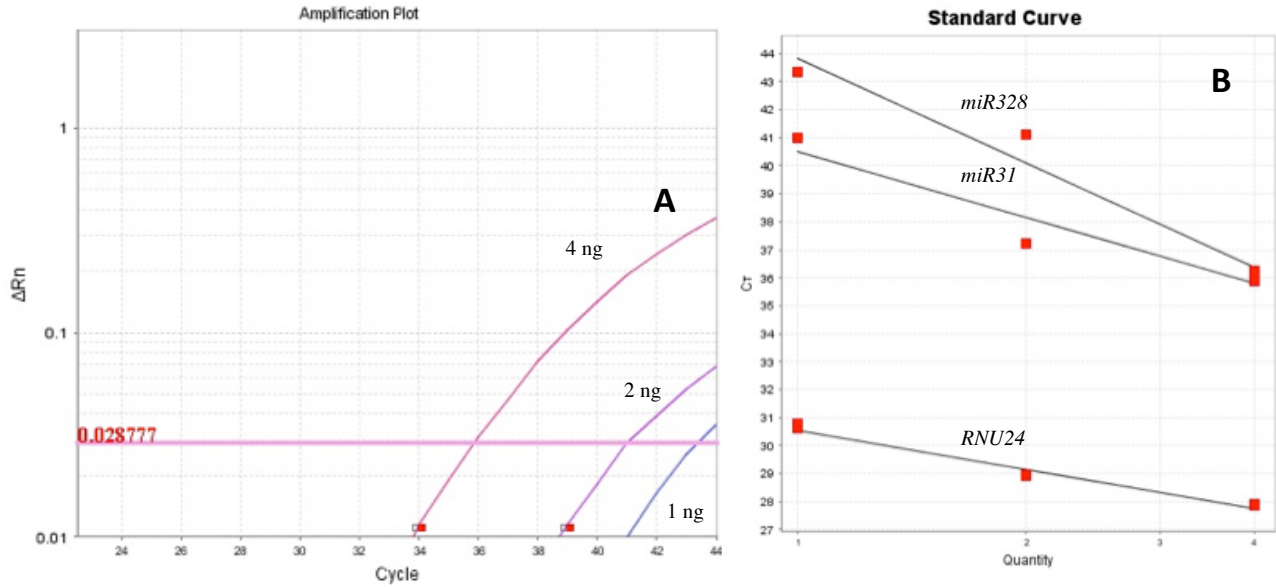


Figure 6. Amplification plots and standard curves for *miR328*. Plots were generated using 1ng, 2ng and 4ng of standard masseter muscle RNA, **A**. Reverse transcribed pool masseter muscle primers for *miR328* **B**. Standard curves for the three microRNA. Horizontal lines indicate the amplification threshold cycle for each plot.

Table 6. Properties of the standard curve for miR328 and miR31

	Slope	Y-Intercept	R ²	Efficiency %
<i>miR328</i>	-3.348	26.99	0.98	98.92
<i>miR31</i>	-7.83	40.52	0.90	34.21
<i>RNU24</i>	-4.70	30.55	0.97	63.36

4.3 Statistical Analysis

Descriptive statistics (average and standard deviation) were calculated for all data sets. The data was then analyzed by gender, vertical dimension, sagittal dimension, TMD, facial symmetry and genotype (Table 7).

Unpaired t-tests were used to determine if the differences in expression levels of *mirR328* between the averages of two groups were significantly different. A p-value ≤ 0.05 was considered to be significant [Appendix D-G]. Analyses between averages of three or more groups were done by a one-way analysis of variance (ANOVA) to determine whether there were any significant differences.

RESULTS

5.1 Results Overview

Muscle RNA was isolated from 62 subjects representing different categories of malocclusion, facial asymmetry, TMD symptoms and *ACTN3 R577X* genotype. Three of 6 miRNAs of interest (*miR193B*, *miR31*, *miR328*), associated with response to ion-channel/transporter functions. Insufficient levels of *miR193B*, *miR31* were detected from our sample (data not shown).

A two-step process was done for quantitation of miRNAs. In step 1, RNA was reverse transcribed in the presence of a specific looped primer for *miR328*. In step 2, primed cDNAs were amplified for quantitation of *miR328* in triplicate, by TaqMan™ RT-PCR methods. Fluorescence data from assays were analyzed by standard curves in each amplification using verified amounts of input cDNA from a pool of all masseter muscle RNA.

MicroRNA 328 was expressed at low levels (pg/ng input RNA) in masseter muscle and values differed between subjects in the separate diagnostic groups (**Table 7**).

Table 7. MiRNA328 Expression in Masseter Muscle In subjects by TMD, ACTN3 R577X Genotype, Sagittal and Vertical Malocclusion.

	Gender		Vertical			Sagittal		Genotype			Asymmetry		TMD	
	Female	Male	Normal	Open	Deep	Class II	Class III	TT	TC	CC	Yes	No	Yes	No
AVG	38.41	43.29	45.02	37.35	38.29	39.48	41.15	42.20	41.03	43.92	42.69	39.42	34.52	42.22
STDEV	16.40	15.81	16.14	16.80	14.86	15.01	19.05	19.97	15.42	10.05	15.80	17.22	11.86	17.41
n	42	20	19	24	19	43	19	13	39	10	21	41	18	44

5.2 Expression of *microRNA 328* by Gender and Sagittal dimension

MicroRNA 328 expression differed overall between sexes (20 males and 42 females), but was not statistically significant ($p=0.3$). Average expression of *microRNA 328* was slightly higher in Class III compared to Class II malocclusions but this difference was not significant ($P=0.7$).

When divided into subgroups of females and males with Class II malocclusion, expression was greater in females, although not at significant levels. Expression of *miR328* in males with Class III malocclusion exceeded that of females, and the difference was statistically significant ($p < 0.04$) (Fig. 7).

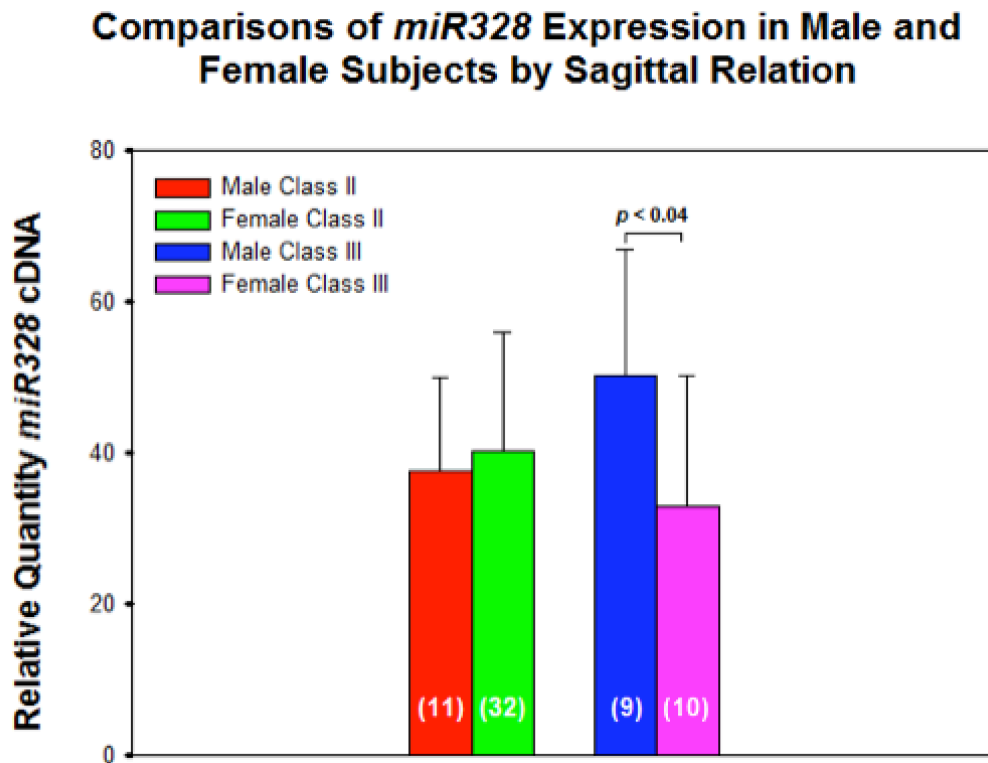


Figure 7. Comparison of Expression of microRNA 328 by Combined Gender and Sagittal Malocclusion Groups

5.3 Expression *microRNA* 328 by TMD-Related Myalgia

All of the 62 patients completed the Jaw, Pain, and Function questionnaire prior to surgery. Eighteen of the 62 subjects had JPF scores ≥ 5 and had signs of TMD. Figure 8 depicts the average expression in muscle, detected by TaqMan RT-PCR, compared by the presence or absence of TMD-related myalgia. Un-paired T-test results found the difference between the groups not quite statistically significant ($p=0.09$). A power analysis determined that a sample size of 60 for each group is needed to detect a statistically significant difference between the two sample means.

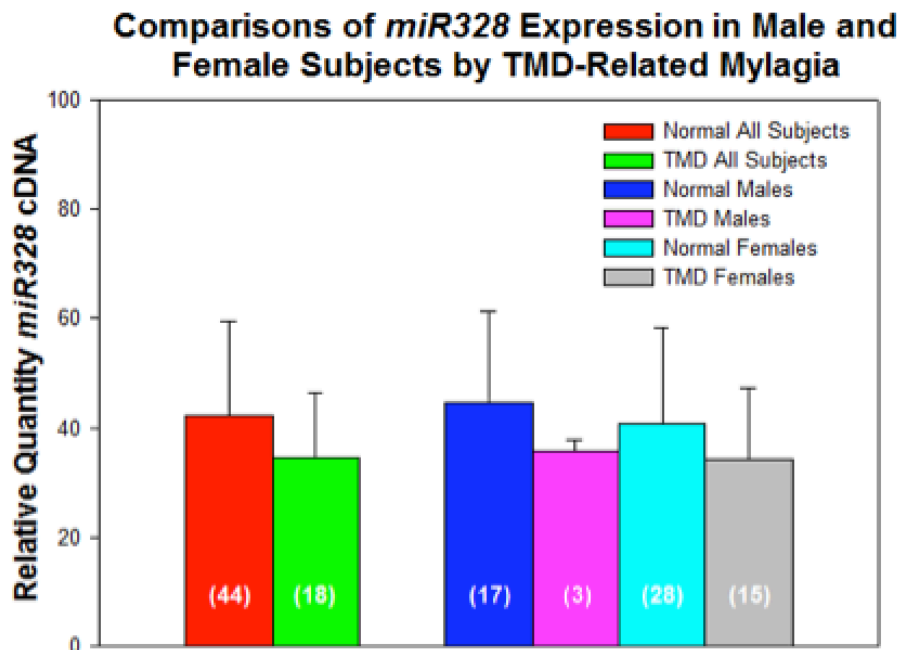


Figure 8. Comparison of Expression of *microRNA* 328 by TMD-Related Myalgia Malocclusion groups.

5.4 Expression microRNA 328 by Vertical dimension Malocclusion

Relative *miR* 328 expression between vertical malocclusion groups was evaluated. Although, higher expression levels were found in subjects with open bite malocclusions, the results on unpaired t-test showed that the differences were not statistically significant for any of the groups (Fig. 9).

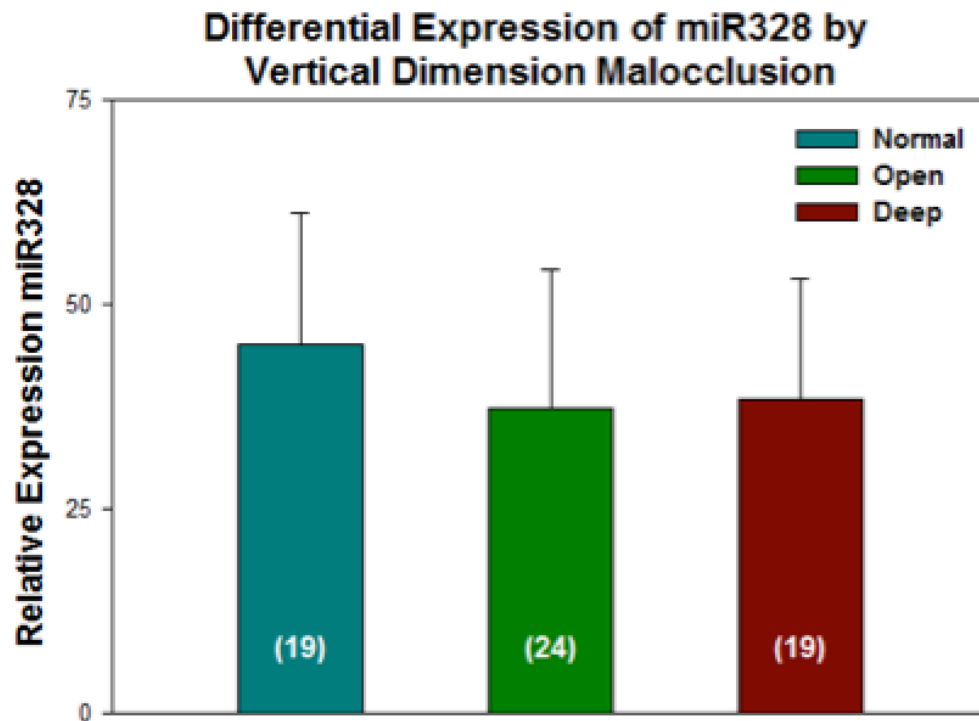


Figure 9. Comparison of Expression of microRNA 328 by Vertical Dimension Malocclusion Groups.

5.5 Expression microRNA 328 by Symmetry and Genotype

Analysis of expression in symmetric versus asymmetric subjects did not find any significance. Expression of *microRNA 328* in *ACTN3 577RR* (mean= 43.93), *ACTN3 577RX* (mean= 41.03) and *ACTN3 577XX* (mean= 42.20) was similar for all 3 groups. No statistical significance differences were found.

The following figure 10 shows the relative expression of *microRNA 328* in all subjects with TMD-related myalgia, Class II malocclusion, Class III malocclusion and, *ACTN3 577RR*, *ACTN3 577RX* and *ACTN3 577XX* genotypes. No statistical significance was found between these groups in the overall sample by ANOVA.

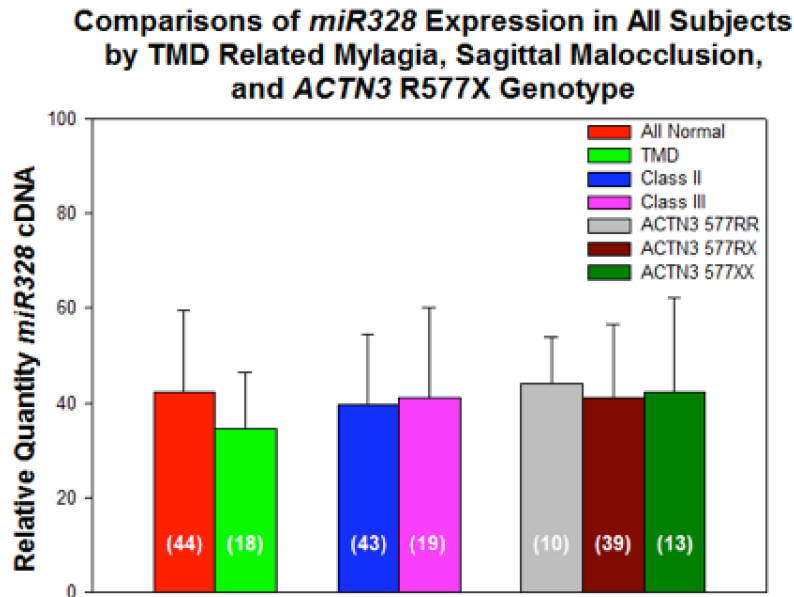


Figure 10. Comparison of Expression of *microRNA 328* by TMD-related myalgia, sagittal malocclusion and *ACTN3* genotype

DISCUSSION

Multiple gene studies have been conducted to determine how genotype and gene expression contribute to the development of non-syndromic dento-facial deformities and TMD. Researchers have found that abnormal growth of the jaws can result in malocclusion leading to facial asymmetries and TMD. Muscle fiber type and alignment have been shown to have an effect on the type of malocclusion developed (Sciote, 2012).

According to Sciote the increase in facial height is inversely proportional to the size and proportion of type-II fibers (fast) in masseter muscle. In patients with mandibular asymmetry, higher type-II fiber occupancies correlate with differences in ramus height, with more fibers seen on the side of the deviation (Sciote et al. 2013). The same study suggests that differences in expression of insulin growth factor and myostatin vary depending on sex and diameter of the fibers.

TMD is the most common chronic oro-facial pain condition with high prevalence of 32% in cases of facial asymmetry (Maixner et al., 2011). The etiology of TMD is multifactorial, but associations have been found between TMD, facial asymmetry and inflammatory/pain related genes. Due to their complex nature, malocclusions can be the result of the interaction of genetic and epigenetic influences on the craniofacial complex. Understanding the combination of the transcription and growth factors acting on bone, teeth, and skeletal muscle will lead to better understanding and treatment of these conditions.

In recent years microRNAs have emerged as important regulators in the mechanism of gene expression. Hypoxia was shown to produce a significant inhibition of *miRNA-328* expression. And insulin-like growth factor-1 receptor and L-type calcium

channel- α 1C have been identified as possible targets for *miRNA-328*. *MicroRNA-328* can suppress the insulin-like growth factor-1 receptor leading to apoptosis of pulmonary arterial smooth muscle cells (Guo et al., 2012) Studies have associated miRNAs injury-response and pain in skeletal muscle, neuronal plasticity, and epigenetic mechanisms for adaptive responses to environmental changes. As a result, microRNAs are seen as potentially useful molecular biomarkers in the diagnosis of disease and therapeutic targets.

Due to the accruing evidence associating miRNAs with important regulatory mechanisms and multiple gene targets this study sought to understand if the expression of *microRNA 328* differed among individuals by gender, *ACTN3 R577X* single nucleotide polymorphism (SNP), TMD-related myalgia, sagittal malocclusion and vertical malocclusion.

TMD has been found to be 1.5-2 times more prevalent in women than men. This increase in prevalence and severity correlates with female reproductive years (Fischer et al., 2008; Von Korff et al., 1988). Women are also more likely to seek treatment than men (LeResche, 1997). In our study, no difference in expression of *microRNA 328* was observed between the female (Mean= 38.42) and male (Mean= 43.30) subjects overall. The number of female (n=42) doubled the number of male (n= 20) subjects in the study.

According to Sciote et al, masseter muscle compressive forces can influence mechano-transduction and modeling of bone and lead to malocclusion. Because the *ACTN3 R577X* single nucleotide polymorphism (SNP) associates with class II and deep bite malocclusions (Zebrick et al, 2014) we wanted to identify whether *microRNA 328*

expression differed among individuals with genotypes for this polymorphism. Among the several diagnostic groups of patients, expression for *microRNA 328* did not differ significantly by unpaired *t*-tests of data between Class II malocclusion subjects. In Class III malocclusion, unpaired *t*-tests showed a statistically significant difference ($p= 0.04$) between *microRNA 328* expression for Class III females (Mean= 32.89) when compared to that of Class III males (Mean= 50.34) subjects. A higher expression of *miR328* in males with Class III malocclusion compared to males with Class II malocclusion was observed, however, this difference was not statistically significant. Whether the higher expression of *microRNA 328* contributes to the increased mandibular length of Class III males is not clear from our study.

Overall, unpaired *t*-test showed that the differences in expression of *microRNA 328* in subjects grouped by vertical dimension, *ACTN3- 5777XX* genotypes, TMD and symmetry were not significant. Although differences in *microRNA 328* between subjects with TMD and without TMD did not reach significance ($p=0.09$), by power analysis a sample size of 60 subjects per group is necessary to detect statistically significant differences between the two groups. The role of microRNAs in pain still needs further investigation but they are likely to cause to changes in protein expression, characteristic of chronic pain. If so, they may become potential targets for analgesic therapies in the future.

Due to the need to obtain masseter muscle samples, the study population was limited to patients undergoing mandibular surgical procedures with no control group of

patients. The very low levels of microRNA (pg/ng input RNA) extracted from the samples increased the study difficulty.

The discovery of microRNAs is recent, and although they have increasingly gained the interest of researchers around the world, more studies are needed to determine their significance as regulators of the multiple developmental, physiological and pathophysiological cell processes.

CONCLUSIONS

- *MicroRNA 328* is present in masseter muscle of our cohort of patients with severe malocclusions undergoing orthognathic surgery.
- Differential expression of *microRNA 328* is significant only between female and male subjects with Class III malocclusion compared to normal dimension and Class II sagittal dimension malocclusion. Expression differences were also found with TMD-related myalgia, and between male Class II and male Class III malocclusion subjects, but a greater number of subjects are needed to determine whether this effect is significant.
- Further studies are needed to identify specific target genes of *microRNA 328* and their underlying role in the development of malocclusion.

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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 : 19/02/2013
Last name : VG	
Identification : 004	
Instructions: Please check the appropriate 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 Subjects Diagnosis

Subject	Sex	Age	Sagittal	Vertical	Asym > 3mm	JPF Score	TMD Signs	Apnea	Race
01	M	17	Class II	Normal	midline R	0	no	no	FR-C
02	W	17	Class II	Open	Minor	2	no	no	FR-C
03	W	41	Class II	Open	Minor	2	no	yes	FR-C
04	W	53	Class III	Open	(PFA)Minor	0	no	no	FR-C
05	W	18	Class III	Deep	midline R	0	no	no	A
06	M	23	Class III	Deep	Minor	3	no	no	FR-C
07	M	45	Class II	Open	No	0	no	yes	FR-C
08	M	29	Class III	Normal	midline L	0	no	no	A
09	W	24	Class II	Deep	midline L	0	no	no	FR-C
010	W	47	Class II	Deep	No	9	yes	no	FR-C
011	M	17	Class II	Normal	No	5	yes	no	FR-C
012	W	24	Class II	Open	Minor	5	yes	no	FR-C
013	W	17	Class II	Open	No	4	no	no	FR-C
014	W	35	Class II	Open	No	5	yes	no	FR-C
015	W	17	Class II	Normal	Minor	3	no	no	FR-C
016	M	17	Class III	Normal	No	1	no	no	FR-C
017	W	14	Class III	Open	Minor	2	no	no	FR-C
018	W	34	Class II	Deep	No	4	no	no	FR-C
019	W	40	Class II	Open	No	3	no	no	FR-C
020	W	30	Class II	Open	Minor	3	no	no	FR-C
021	M	26	Class II	Open	midline R	0	no	no	FR-C
022	W	45	Class II	Normal	No	1	no	no	FR-C
023	W	20	Class II	Open	No	2	no	no	A
024	M	18	Class III	Open	Minor	1	no	no	FR-C
025	W	38	Class III	Open	midline L	2	no	no	FR-C
026	W	31	Class II	Deep	No	17	yes	no	FR-C
027	W	15	Class II	Deep	Minor	0	no	no	FR-C
028	W	16	Class II	Normal	Minor	0	no	no	FR-C
029	M	36	Class II	Deep	Minor	3	no	no	FR-C
030	W	24	Class II	Open	midline	6	yes	no	FR-C

030	W	24	Class II	Open	midline L	6	yes	no	FR-C
031	M	20	Class III	Open	midline left	1	no	no	FR-C
032	M	15	Class II	Normal	Minor	0	no	no	FR-C
033	W	20	Class II	Open	midline R	14	yes	no	FR-C
034	W	15	Class II	Open	midline R	5	yes	no	FR-C
035	W	16	Class III	Open	No	2	no	no	FR-C
036	W	41	Class II	Open	(PFA) midline L	14	yes	no	FR-C
037	W	16	Class II	Normal	No	1	no	no	Caucasian
038	W	15	Class II	Deep	Minor	6	yes	no	Caucasian
039	W	28	Class II	Open	5-L	3	no	no	FR-C
040	W	16	Class III	Deep	minor	7	yes	no	FR-C
041	W	15	Class II	Deep	no	1	no	no	Parsee
042	M	21	Class II	Deep	3-R	13	yes	no	FR-C
043	W	34	Class III	Deep	minor	1	no	no	A
044	M	19	Class III	Normal	Minor	2	no	no	FR-C
045	W	16	Class II	Deep	Minor	0	no	no	FR-C
046	M	16	Class III	Normal	3-L	5	no	no	FR-C
047	W	18	Class II	Open	No	9	yes	no	FR-C
048	W	34	Class II	Open	3-L	18	yes	no	FR-C
049	M	16	Class II	Normal	Minor	1	no	no	FR-C
050	W	15	Class II	Normal	Minor	0	no	no	FR-C
051	M	17	Class III	Normal	No	0	no	no	FR-C
052	W	23	Class III	Deep	No	9	yes	no	NA
053	M	16	Class II	Deep	No	0	no	no	FR-C
054	M	17	Class II	Normal	No	13	yes	no	FR-C
055	M	17	Class III	Open	4-R	4	no	no	FR-C
056	W	18	Class II	Normal	5-L	2	no	no	FR-C
057	W	18	Class II	Deep	No	0	no	no	FR-C
058	M	16	Class II	Deep	4-R	0	no	no	FR-C
059	W	17	Class III	Normal	5-L	10	yes	no	FR-C
060	W	18	Class II	Normal	No	10	yes	no	FR-C
061	W	17	Class III	Deep	No	2	no	no	FR-C

APPENDIX C

Summary of MicroRNA 328 Expression Data

No	Sex	Age	Sagittal	Vertical	Asym > 3mm	JPF Score	TMD Signs	Apnea	Race	Genotype	miR328
01	W	16	Class II	Open	midline L	0	no	no	FR-C	TT	—
02	M	17	Class II	Normal	midline R	0	no	no	FR-C	CC	60.627
03	W	17	Class II	Open	Minor	2	no	no	FR-C	TC	45.928
04	W	41	Class II	Open	Minor	2	no	yes	FR-C	TC	27.643
05	W	53	Class III	Open	(PFA) Minor	0	no	no	FR-C	TT	61.091
06	W	18	Class III	Deep	midline R	0	no	no	A	CC	51.793
07	M	23	Class III	Deep	Minor	3	no	no	FR-C	TT	41.947
08	M	45	Class II	Open	No	0	no	yes	FR-C	TT	38.898
09	M	29	Class III	Normal	midline L	0	no	no	A	TC	37.790
010	W	24	Class II	Deep	midline L	0	no	no	FR-C	TC	36.266
011	W	47	Class II	Deep	No	9	yes	no	FR-C	TC	49.787
012	M	17	Class II	Normal	No	5	yes	no	FR-C	TT	33.397
013	W	24	Class II	Open	Minor	5	yes	no	FR-C	CC	43.424
014	W	17	Class II	Open	No	4	no	no	FR-C	TC	50.902
015	W	35	Class II	Open	No	5	yes	no	FR-C	CC	35.866
016	W	17	Class II	Normal	Minor	3	no	no	FR-C	TC	43.277
017	M	17	Class III	Normal	No	1	no	no	FR-C	TT	76.378
018	W	14	Class III	Open	Minor	2	no	no	FR-C	TC	24.744
019	W	34	Class II	Deep	No	4	no	no	FR-C	TC	48.373
020	W	40	Class II	Open	No	3	no	no	FR-C	TT	9.150
021	W	30	Class II	Open	Minor	3	no	no	FR-C	CC	26.832
022	M	26	Class II	Open	midline R	0	no	no	FR-C	TC	62.291
023	W	45	Class II	Normal	No	1	no	no	FR-C	TC	56.431
024	W	20	Class II	Open	No	2	no	no	A	TC	0.507
025	M	18	Class III	Open	Minor	1	no	no	FR-C	TC	19.399
026	W	38	Class III	Open	midline L	2	no	no	FR-C	TT	13.415
027	W	31	Class II	Deep	No	17	yes	no	FR-C	TC	37.736
028	W	15	Class II	Deep	Minor	0	no	no	FR-C	TC	27.730
029	W	16	Class II	Normal	Minor	0	no	no	FR-C	TT	36.248
030	M	36	Class II	Deep	Minor	3	no	no	FR-C	TC	20.179
031	W	24	Class II	Open	midline L	6	yes	no	FR-C	TC	26.867
032	M	20	Class III	Open	midline left	1	no	no	FR-C	CC	46.580
033	M	15	Class II	Normal	Minor	0	no	no	FR-C	TC	32.365
034	W	20	Class II	Open	midline R	14	yes	no	FR-C	TT	34.727

035	W	15	Class II	Open	midline R	5	yes	no	FR-C	TC	29.270
036	W	16	Class III	Open	No	2	no	no	FR-C	TC	45.327
037	W	16	Class III	Deep	midline R	4	no	no	FR-C	TC	—
038	W	41	Class II	Open	(PFA) midline L	14	yes	no	FR-C	TC	29.991
039	W	16	Class II	Normal	No	1	no	no	Caucasi an	TC	41.189
040	W	15	Class II	Deep	Minor	6	yes	no	Caucasi an	TC	46.511
041	W	28	Class II	Open	5-L	3	no	no	FR-C	TC	63.641
042	W	16	Class III	Deep	minor	7	yes	no	FR-C	TC	33.980
043	W	15	Class II	Deep	no	1	no	no	Parsee	TT	48.582
044	M	21	Class II	Deep	3-R	13	yes	no	FR-C	TC	38.472
045	W	34	Class III	Deep	minor	1	no	no	A	CC	42.336
046	M	19	Class III	Normal	Minor	2	no	no	FR-C	TC	61.387
047	W	16	Class II	Deep	Minor	0	no	no	FR-C	TC	77.220
048	M	16	Class III	Normal	3-L	5	no	no	FR-C	TC	42.493
049	W	18	Class II	Open	No	9	yes	no	FR-C	TC	50.309
050	W	34	Class II	Open	3-L	18	yes	no	FR-C	TC	51.063
051	M	16	Class II	Normal	Minor	1	no	no	FR-C	TC	36.321
052	W	15	Class II	Normal	Minor	0	no	no	FR-C	TT	75.322
053	M	17	Class III	Normal	No	0	no	no	FR-C	TC	68.480
054	W	23	Class III	Deep	No	9	yes	no	NA	CC	2.054
055	M	16	Class II	Deep	No	0	no	no	FR-C	TC	31.422
056	M	17	Class II	Normal	No	13	yes	no	FR-C	TC	35.401
057	M	17	Class III	Open	4-R	4	no	no	FR-C	CC	58.552
058	W	18	Class II	Normal	5-L	2	no	no	FR-C	TT	37.346
059	W	18	Class II	Deep	No	0	no	no	FR-C	TC	34.251
060	M	16	Class II	Deep	4-R	0	no	no	FR-C	TC	23.591
61	W	17	Class III	Normal	5-L	10	yes	no	FR-C	TC	18.743
62	W	18	Class II	Normal	No	10	yes	no	FR-C	CC	23.881
63	W	17	Class III	Deep	No	2	no	no	FR-C	TT	35.413
64	W	16	Class III	Open	No	14	yes	no	FR-C	TT	—
65	W	18	Class II	Normal	3-L	2	no	no	FR-C	TC	38.399

APPENDIX D
Unpaired T-Tests Results: Vertical Dimension

Vertical	n	Mean	SD	t	df	p	95% Confidence interval
Open	24	37.35114063	16.80493747				
Deep	19	38.29753751	14.86492572				
Total	43			0.1928	41	0.848	-10.857 to 8.965

Vertical	n	Mean	SD	t	df	p	95% Confidence interval
Normal	19	45.02550601	16.14141028				
Deep	19	38.29753751	14.86492572				
Total	38			1.3365	36	0.1898	-3.481 to 16.937

Vertical	n	Mean	SD	t	df	p	95% Confidence interval
Open +							
Deep	43	37.76931599	15.9837088				
Normal	19	45.02550601	16.14141028				
Total	62			1.6431	60	0.1056	-1.577 to 16.0899

APPENDIX E
Unpaired T-Tests Results: Genotype

Genotype	n	Mean	SD	t	df	p	95% Confidence interval
TC	39	41.0384	15.4247				
CC	10	43.923	10.0555				
Total	49			0.5593	47	0.5786	-13.260 to 7.491

Genotype	n	Mean	SD	t	df	p	95% Confidence interval
TT	13	42.2088	19.9729				
CC	10	43.923	10.0555				
Total	23			0.2474	21	0.807	-16.121 to 12.693

Genotype	n	Mean	SD	t	df	p	95% Confidence interval
TT	13	42.2088	19.9729				
TC	39	41.0384	15.4247				
Total	52			0.2198	50	0.827	-9.526 to 11.867

APPENDIX F
Unpaired T-Tests Results: TMD-related Myalgia and Symmetry

<i>Group</i>	<i>n</i>	<i>Mean</i>	<i>SD</i>	<i>t</i>	<i>df</i>	<i>p</i>	<i>95% Confidence interval</i>
Symm	41	39.42795416	17.22226086				
Asymm	21	42.69111402	15.80178314				
Total	62			0.7255	60	0.471	-12.260 to 5.734

<i>Group</i>	<i>n</i>	<i>Mean</i>	<i>SD</i>	<i>t</i>	<i>df</i>	<i>p</i>	<i>95% Confidence interval</i>
(-) TMD	44	42.2290292	17.41213455				
(+) TMD	18	34.52710652	11.86026592				
Total	62			1.7167	60	0.0912	-1.272 to 16.676

APPENDIX G
Unpaired T-Tests Results: Gender and Sagittal Malocclusion

Sagittal	n	Mean	SD	t	df	p	95% Confidence interval
Class II	43	39.48031715	15.01417911				
Class III	19	41.15324023	19.05614211				
Total	62			0.3718	60	0.7113	-10.672 to 7.326

Gender	n	Mean	SD	t	df	p	95% Confidence Interval
Female	42	38.41868462	16.40839155				
Male	20	43.2990224	15.81012164				
Total	62			1.1074	60	0.27	-3.94 to 13.69

Female	n	Mean	SD	t	df	p	95% Confidence interval
Class II	11	37.54276701	12.54723488				
Class III	9	50.33444565	16.52432864				
Total	20			1.9694	18	0.0645	-26.437 to 0.853

Class II	n	Mean	SD	t	df	p	95% Confidence interval
Female	32	40.1464	15.7183				
Male	11	37.5428	12.5472				
Total	43			0.4964	41	0.6223	-13.196 to 7.989

Class III	n	Mean	SD	t	df	p	95% Confidence interval
Female	10	32.89015534	17.32246243				
Male	9	50.33444565	16.52432864				
Total	19			2.2397	17	0.04	1.011 to 33.877

Female	n	Mean	SD	t	df	p	95% Confidence interval
Class II	32	40.14635002	15.71826691				
Class III	10	32.89015534	17.32246243				
Total	42			1.2446	40	0.2205	-4.527 to 19.039