Masseter muscle gene expression in relation to various craniofacial deformities: A genotype-phenotype study

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Dedication

To my beloved parents

My sisters:
Ruba
Anadel
Ranad

My brother:
Mohamed

You are the greatest treasure of my life and without your love, support and prayers I would have never produced this work

ABSTRACT

Craniofacial form is defined by a number of factors. A major contributor is the jaw musculature especially of the masseter muscle, as differences in transcription and translation of various genes have been documented from this tissue. Up to this point however, no reliable biological predictors of form have been identified.

The aims of this study were therefore, to describe the transcriptome of the masseter muscle using microarray technology and to establish and correlate the expression levels of potential candidate and known "informative" genes in masseter muscle with selected clinical, radiographic and dental features of subjects with a variety of craniofacial morphologies.

A total of 29 patients (18 deformity and 11 control) were selected from the orthodontic/orthognathic clinics at the Eastman Dental and Whipps Cross Hospitals, London, and Riyadh Military Hospital, Saudi Arabia.

Microarray results indicated five "novel" genes not previously reported in relation to the masseter muscles of subjects with variable craniofacial morphologies. Two genes (KIAA1671 and DGCR6) were down-regulated in long face patients, one (SERGEF) was down-regulated in Class III patients and one (LOC730245) was up-regulated in Class II long faces and in all Class III subjects, compared to controls. Another gene (NDRG2) was down-regulated in Class II compared to Class III individuals. Subsequent quantitative Reverse Transcriptase PCR results strongly confirmed that the "novel" gene SERGEF was down-regulated in relation to the clinical, dental and radiographic features of subjects with Class III appearance. SERGEF gene had a positive relationship to the number of dental occlusal contacts and ANB angle. The "informative" gene *MHC*7 was strongly related to both vertical and horizontal facial deformities.

These data suggest that the expression profiles of a number of genes can be analysed and used to make assessments as to their role in the primary aetiology and successful or unsuccessful treatment of patients with specific craniofacial morphologies.

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DECLARATION

I, Hadwah AbdelMatloub Moawad confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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LIST OF ABBREVIATIONS

ADP	Adenosine DiPhosphate
ANB	A-point-Nasion-B-point angle
AOB	Anterior Open Bite
ATP	Adenosine TriPhosphate
BCB	Bloomsbury Centre for Bioinformatics
Ca	Calcium
cDNA	Complementary DeoxyriboNucleic Acid
cRNA	Complementary RiboNucleic Acid
CSA	Cross Sectional Area
CT	Computerised Tomography
Ct	Crossing threshold
DB	Deep Bite
DelGEF	Deafness Locus associated putative Guanine-nucleotide Exchange Factor
DGCR6	DiGeorge syndrome Critical Region gene 6
DNA	DeoxyriboNucleic Acid
dNTP	DeoxyriboNucloside TriPhosphate
ECM	Extra Cellular Matrix
EDH	Eastman Dental Hospital
EMG	Electromyography
Ex	Efficiency test
FGFR	Fibroblast Growth Factor Receptor
Fu	Fluorescence
GAPDH	Glyceraldehyde-3-Phosphate DeHydrogenase
GCOS	Gene Chip Operating Software
GCRMA	Guanine-Cytosine Robust Multi Array
GSP	Gene Specific Primers
h	Hour
HG	Human Genome
IGF	Insulin-like Growth Factor
IVT	*In-Vitro* Transcription
Kb	Kilo Base
KIAA	Genes with unidentified protein function, investigated by the Kazusa DNA Research Institute
KSA	Kingdom of Saudi Arabia
LAFH	Lower Anterior Face Height
LOC	Locus
m	Minute
MF	Magnification factor
Mg	Magnesium
mg	Milligram
MIAME	Minimum Information About a Microarray Experiment
mm	Millimetre
MM	Mis-Match probe
MMa	Maxillary-Mandibular angle
MMP	Matrix MetalloProteinases
MRI	Magnetic resonance Image
mRNA	Messenger Ribonucleic Acid
MYH	Myosin Heavy Chain gene
MyHC	Myosin Heavy Chain protein
MyHCemb	Myosin Heavy Chain Embryonic protein
MyHCneo	Myosin Heavy Chain Neonatal protein
NDRG2	Neuroblastomas- myelocytomatosis Downstream Regulated Gene 2
nm	Nanometre

nt	Nucleotide
OD	Optical Density
PCR	Polymerase Chain Reaction
PM probe	Perfect-Match probe
REC	Research Ethics Committee
RH	Ramus Height
RMH	Riyadh Military Hospital
RNA	RiboNucleic Acid
qRT-PCR	Quantitative Reverse-Transcriptase Polymerase Chain Reaction
rRNA	Ribosomal RiboNucleic Acid
RT	Reverse Transcription
s	Second
S	Subunit
SAPE	Streptavidin-PhycoErythrin
SD	Standard Deviation
SDD	Standard Deviation of the Difference
SERGEF	Secretion Regulating Guanine Nucleotide Exchange Factor
SNA	Sella-Nasion-A-point angle
SNB	Sella-Nasion-B-point angle
SN-MP	Sella-Nasion-Mandibular Plane angle
SN-MxP	Sella-Nasion-Maxillary Plane angle
SNP	Single Nucleotide Polymorphism
SPSS	Statistical Package for Social Sciences
TAFH	Total Anterior Face Height
TIMP	Tissue Inhibitors of Metalloproteinases
TPFH	Total Posterior Face Height
UK	United Kingdom
WCH	Whipps Cross Hospital

Chapter 1. Background

1.1. INTRODUCTION

Structural (Boyd et al., 1989) and functional (Hunt and Cunningham, 1997) variations of the masseter muscle between individuals with various facial morphologies (Kiliaridis and Kalebo, 1991; Ariji et al., 2000) are well documented. Recent masseter muscle research in the orthodontic field has been driven by the hypotheses as to whether these differences could be detected on a transcriptome level, to be used, alongside the clinical diagnosis, as markers for specific craniofacial deformities (Nelson-Moon et al., 1998) or as predictors for success or relapse following treatment (Harzer et al., 2007). In an attempt to answer these questions, scientists have proposed a set of candidate genes which have been reported in association with the development (Butler-Brown et al., 1988), contraction (Eriksson and Thornell, 1983) regeneration (Singh et al., 2000) or adaptation (Auluck et al., 2005) of the masseter muscle. Out of the large number of human genes (Rabinowicz et al., 2000), very few genes have been selected and the results in relation to craniofacial deformities have been variable. Up to date no reliable predictors have been identified (Suchak et al., 2009).

The aims of this chapter are therefore; to provide a general background of the structure and function of the masseter muscle in both normal and abnormal craniofacial morphologies, to give an up to date summary of the masseter muscle genes that have been tested in relation to craniofacial discrepancies, whilst the final section will elucidate the scope of new gene expression technologies, which have not been implemented previously in masseter muscle research and which could provide valuable transcriptome data.

1.2. THE MASSETER MUSCLE

The masseter muscle is one of the muscles of mastication that has been investigated extensively in humans. This has been due to its close relation to the mandible and its ease of accessibility (Yonemitsu et al., 2007). It is composed of two portions, the deep and the superficial bellies (Figure 1.1).

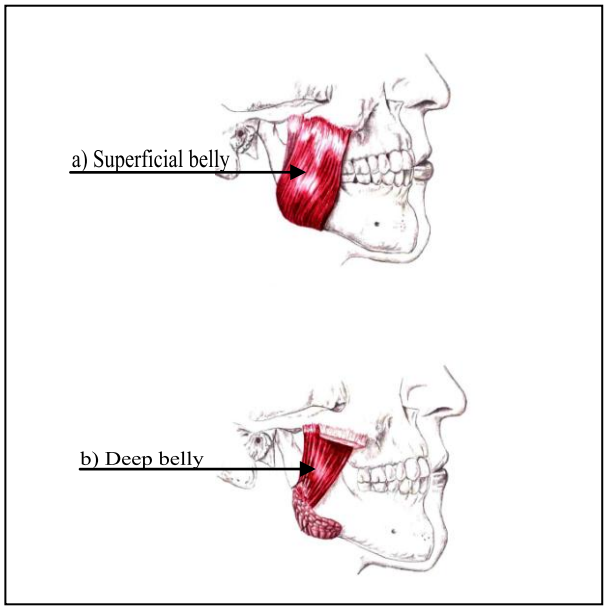


Figure 1.1: Anatomy of the masseter muscle. a) *The superficial belly arises from the zygomatic process of the maxilla as well as the zygomatic arch and runs downwards in a diagonal direction to be inserted onto the lower lateral border of the ramus of the mandible and the gonial angle.* ***b)*** *The deep belly arises from the medial surface of the zygomatic arch and runs downwards and backwards to be inserted onto the upper half of the ramus of the mandible (adapted from Biel, 2005).*

1.2.1. GENERAL MASSETER MUSCLE STRUCTURE AND FUNCTION

1.2.1.1. Intrauterine growth

Migration of cells originating from the paraxial mesoderm into the first branchial arch gives rise to the muscles of mastication (temporalis, masseter, medial and lateral pterygoid), anterior belly of the digastric, mylohyoid, tensor tympani and tensor veli palatini. Primitive craniofacial muscle cells are evident at the 8^{th} - 9^{th} week of intrauterine life and are called myoblasts. Following differentiation, the myoblasts elongate and fuse together to form multinucleated cylindrical myotubes. The mature forms of the myotubes are called muscle fibres. A group of muscle fibres are called fascicles, and a group of fascicles forms the whole muscle (Figure 1.2). The highly specialised processes of muscle cell proliferation (Henriquez et al., 2002), differentiation (Osses and Brandan, 2002) and fusion (Carrino eta al., 1999) into muscle fibres and fascicles have been found to be largely influenced by the surrounding connective tissue which is called the extracellular matrix (ECM).

The ECM is organized into three main layers, endomysium, perimysium and epimysium (Figure 1.2), and is mainly a collection of collagens, glycoproteins and proteoglycans (reviewed in Velleman, 1999). This collection forms a dynamic network that surrounds and connects the different layers of the muscle (Lewis et al., 2001). Not only does the composition of the ECM affect the structural and functional state of the muscular tissue, but also the interaction and adhesion between the ECM and the muscle fibres (Grounds et al., 2005). It also contributes to the integrity of the muscle (Stetler-Stevenson, 1996).

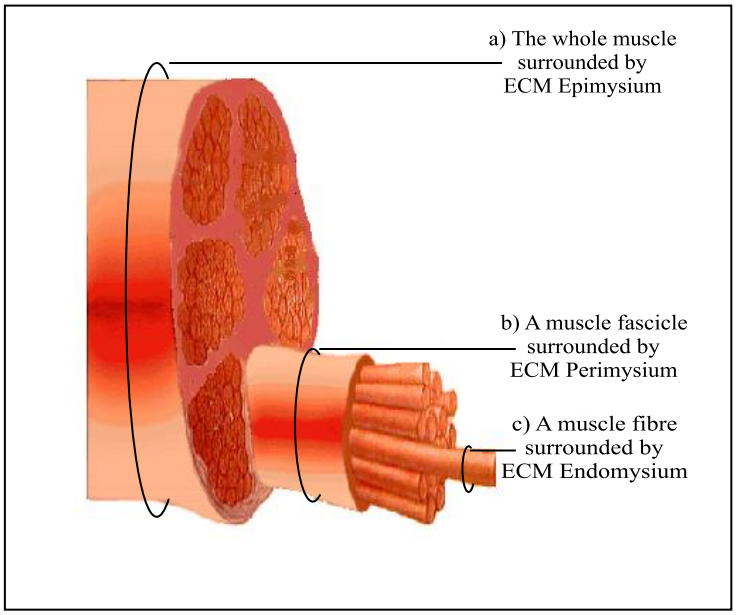


Figure 1.2: General muscle structure. a) *The epimysium is the outermost sheet that surrounds the whole muscle.* ***b)*** *The perimysium is the intermediate layer of the ECM that covers each fascicle.* ***c)*** *The endomysium is the innermost layer of the ECM and surrounds each fibre. ECM, extracellular matrix (adapted and modified from www.dkimages.com).*

By the 14th - 15th week of intrauterine life, all craniofacial muscles have established their sites of origin and insertion (Sperber, 1989). However, the contractile properties of the muscle fibres are not evident until the 22nd week of gestation (Ringqvist et al., 1977)

1.2.1.2. Contraction of muscle fibres

The contraction of a single muscle fibre is brought about by the motor unit within each myofibril, called the sarcomere. The sarcomere is composed of two types of filaments; the thin actin and thick myosin protein filaments. The myosin filament comes in a greater variety than the actin filament; it is composed of two heavy chains and four light

chains, while the actin has only two polypeptide chains namely, troponin and tropomyosin (Figure 1.3).

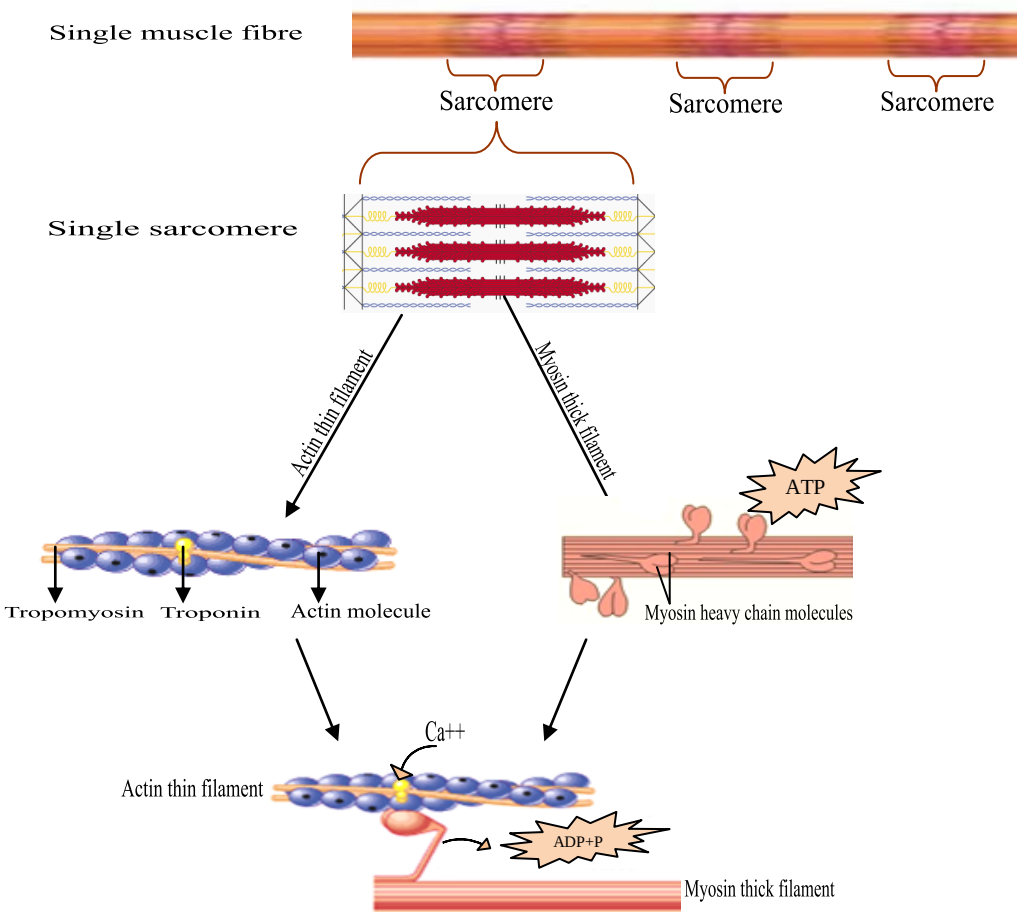


Figure 1.3: The motor unit in muscle fibres. *Once there is an action stimulus, calcium ions binds to troponin, which subsequently changes the tropomyosin configuration allowing the interaction between both actin and myosin molecules, causing contraction and shortening of the muscle fibre. This contraction requires energy which is generated by the myosin heavy chain (MyHC) heads which contains an adenosine triphosphate (ATP) site and an enzyme called ATPase which hydrolyses the ATP into adenosine diphosphate (ADP) and provides the required energy. P: Released phosphate (adapted and modified from http://www.octc.kctcs.edu).*

1.2.1.3. Postnatal growth and development

Generally, postnatal growth and development of the muscular tissue occurs via the elongation and increase in diameter of the muscle fibres (hypertrophy). Soon after birth, all muscle fibres have gained their contractile properties. A fully mature masticatory muscle often contains different subpopulations of slow, fast and hybrid muscle fibres (Ringqvist et al., 1982). However, depending on functional demands, the muscles of mastication can undergo a transitional state recruiting different muscle fibres

(Kobayashi et al., 2001) and various contractile protein isoforms (Boyd et al., 1989). This heterogeneity in muscle structure may allow for adaptability to different developmental (Monemi et al., 1996; Raadsheer et al., 1996) hormonal (Eason et al., 2000) and functional demands (Auluck et al., 2005) during postnatal life.

Postnatal masseter muscle development has been largely related to mandibular growth (Yonemitsu et al., 2007). Both the deep and the superficial bellies of the masseter muscle are inserted onto the posterior surface of the ramus and mandibular gonial angle, respectively. The posterior surface of the mandible undergoes progressive postnatal remodelling, and therefore the masseter muscle is in a continuous alert state to readjust its site of insertion and so adapt to postnatal mandibular changes (Sperber, 1989).

1.2.1.4. Masseter muscle fibre types

Several methods have been employed to classify muscle fibres (e.g. histochemical typ I, IIA and IIB fibres) (Brook and Kaiser, 1970; Barnard et al., 1971). However, with the evolution of science, new classifications have been implemented using mainly molecular genetic techniques. This scheme includes the transcriptome (gene expression) and proteomic (protein) expression properties of the myosin heavy chain (MyHC) proteins (Gorza, 1990; Smerdu *et al.,* 1994; Termin et al., 1989). The human masseter muscle has been reported to have various MyHC proteins encoded by various myosin heavy chain genes (*MYH*) (Table 1.1).

Table 1.1: Myosin heavy chain genes and endcoded proteins that have been used to classify human masseter muscle fibres.

MyHC protein isoforms	Encoding gene*	Description
MyHC I (Smerdu et al., 1994) MyHC IIa (Eriksson and Thornell, 1983) (Ringqvist et al., 1982)	*MYH7* *MYH2*	-Reported in slow contracting muscle fibres - Reported in fast contracting muscle fibres -Both I and IIa MyHC isoforms have also been present in hybrid fibres where the masseter muscle can shift from slow to fast contracting proteins or *vice versa*, depending on functional demands
MyHC IId/x (Smerdu et al., 1994)	*MYH1*	-Reported in faster contracting muscle fibres as well as hybrid fibres with MyHC IIa but not MyHC I
MyHC α –cardiac (d'Albis et al., 1993)	*MYH6*	-Expressed in both the masseter muscle as well as the cardiac muscle and was, therefore, called alpha cardiac protein -Co-expressed in hybrid fibres
MyHC embryonic (Monemi et al., 1996) MyHC neonatal (Sciote et al., 2003)	*MYH3* *MYH8*	-Both proteins are evident in the pre- and postnatal life of the masseter muscle as well as other muscles of mastication, which tend to be replaced by fast or slow contracting isoforms in the postnatal form of other skeletal muscles such as limb muscle -Both proteins have been co-expressed in hybrid fibres

MyHC: Myosin heavy chain protein. *MYH*: Myosin heavy chain gene. *(Yoon et al., 1992; Shrager et al., 2000)

1.2.1.5. Masseter muscle extracellular matrix

The ECM is a versatile structure which can undergo continuous remodelling via degradation and deposition. The synchronized rhythm between these two processes and the ability of the ECM proteins to perform these tasks under normal physiologic or pathologic conditions is referred to as ECM turnover (Stetler-Stevenson, 1996). Fibronectin, which is one of the non-collagenous glycoproteins, has been reported to regulate deposition (Sottile and Chandler, 2005), while the enzyme family of matrix metalloproteinases (MMPs) have been considered to control degradation during pathologic conditions (Stetler-Stevenson, 1996). Expression of the MMP enzymes has also been observed during normal biological conditions such as cell migration and morphogenesis (Werb and Chin, 1998). Up- or down-regulation of the MMPs is said to

be regulated by a group of other enzymes namely, the tissue inhibitor of metalloproteinases (TIMPs) (Lewis et al., 2001).

The masseter muscle has been reported with an embryonic type of fibronectin which was not present in somatic cells (Price *et al.*, 1998), as well as some of the MMPs and TIMPs such as MMP1, MMP2, MMP9, and the TIMP2 (Singh *et al.*, 2000; Tippett *et al.*, 2008).

Both muscle fibres (Pavlath et al., 1998) and the ECM (Goldspink, 1998) play an important role in muscle adaptation and regeneration. Inability of the masseter muscle to attain normal growth or undergo remodelling has been reported to affect craniofacial morphology (Lowe, 1980; Schessl et al., 2006).

1.3. CRANIOFACIAL MORPHOLOGY

Craniofacial morphology is orchestrated by both skeletal structures and muscular tissue (Lowe, 1980; Helm and German, 1996; Bresin and Kiliaridis, 2002; Satiroğlu et al., 2005). Of the skeletal structures, the cranial base, the maxilla and the mandible have been found to contribute largely to variations in craniofacial morphology. The following sections will briefly describe the normal intrauterine growth and postnatal development of the cranial base, the maxilla and the mandible.

1.3.1. THE CRANIAL BASE

1.3.1.1. Intrauterine growth

The cranial base appears in the 8th week during intrauterine life as four midline and two lateral structures which later form the frontal, ethmoid, sphenoid, occipital, and the temporal bones. These bones are formed through endochondral ossification and are united via synchondroses. The mid-sphenoidal synchondrosis divides the cranial base into anterior and posterior sections. Each section is derived from a distinct embryonic origin. The anterior cranial base is derived from migration of the neural crest cells, while the posterior portion is derived from cells of the paraxial mesoderm (Sperber, 1989).

1.3.1.2. Postnatal growth and development

The general trend of postnatal growth and development of the cranial base is seen as a downward and forward movement which has been mainly attributed to downward development of the brain (Moss, 1968) and forward displacement of the spinal column (Björk, 1950). The anterior cranial base is determined radiographically as a line extending from nasion to the mid-point of sella turcica. A line from sella turcica extending posteriorly to basion determines the posterior cranial base (Björk, 1955). The maxilla is attached to the anterior part while the mandible articulates with the posterior section. Discrepancies of the anterior cranial base can affect mid facial development (Ma and Lozanoff, 1996), whereas posterior cranial base discrepancies may have a marked influence on the position of the mandible (Ngan et al., 1997).

1.3.2. THE MAXILLA

1.3.2.1. Intrauterine growth

Both maxillary processes of the 1st brachial arch give rise to the maxilla and the secondary palate, which unite at the middle at the median palatal suture. The maxilla grows by intramembranous ossification that begins during the 7th week of intrauterine life.

1.3.2.2. Postnatal growth and development

Postnatal growth and development of the maxilla is seen in vertical, horizontal and transverse dimensions. Both vertical and horizontal development of the maxilla are affected by downward and forward displacement of the cranial base. During this displacement, the sutures between the maxilla and the cranial base tend to open up and the space is filled with bone deposits which contribute to vertical maxillary growth (Björk, 1955). Horizontal growth of the maxilla is also governed by downward and forward movement of the cranial base, as dramatic maxillary anterior bone resorption is evident and incremental bone deposition at the posterior tuberosity area occurs. This is to accommodate the eruption of the permanent molar teeth later in life (Björk and Skieller, 1977). Transverse development of the maxilla is brought about by an increase in width of the median palatal suture which is more evident in the molar region than the anterior mid-canine area (Björk and Skieller, 1977; Korn and Baumrind, 1990). Sutural

growth in the transverse and horizontal planes is completed around the age of 17 years (Björk, 1966), while vertical growth is still evident at the age of 21 years (Björk and Skieller, 1977). Dimensional variation of the maxilla in the vertical (Schendel et al., 1976), horizontal (Guyer et al., 1986) and transverse (Wolford and Stevao, 2002) planes is seen in patients with craniofacial deformities.

1.3.3. THE MANDIBLE

1.3.3.1. Intrauterine growth

The mandible appears as two elevations or a bilateral structure in the 6th week of intrauterine life. The paired bony structures of the mandible are joined together in the midline by a fibrocartilage in the mandibular symphysis during intrauterine life. By the end of the 1st year of foetal life both halves have united by endochondral ossification. The mandible develops entirely by intramembranous ossification and grows by direct surface apposition, resorption and remodelling, except for the condyle which undergoes endochondral ossification and grows by synchondroses (Sperber, 1989).

1.3.3.2. Postnatal growth and development

Postnatal growth and development of the mandible is more complicated than for other craniofacial structures. This has been explained by Enlow and Harris (1964) in a detailed study of 25 specimens of human mandibles aged 4 -12 years. They found that during growth, each part of the mandible (coronoid process, condylar process, the neck of the condyle, the ramus, the body of the mandible and the chin) undergoes remodelling to maintain the size and shape of the mandible. However, both the condyle (Marshall, 1958) and the posterior surface of the ramus were more likely to be mandibular growth sites (Proffit and Fields, 2000) and to undergo progressive remodelling until later in life (Enlow and Harris, 1964). The mandible grows in transverse, horizontal, and vertical directions. Transverse growth ceases first, prior to puberty, followed by horizontal growth at the age of 14-15 years in females and two years later in males (Hellman, 1927; Marshall, 1958). Similar to maxillary growth, the vertical growth of the mandible continues after horizontal growth has slowed or ceased and may extend until the age of 40 years (Lewis and Roche, 1988). This is to match the general vertical growth of the face that continues throughout life (Hellman, 1927). Both

the size (Sanborn, 1955) and the position of the mandible (Björk, 1950; Kao et al., 1995) are affected in patients with variable craniofacial discrepancies.

One of the unique postnatal developmental features of the mandible that can cause large variations in vertical craniofacial features is mandibular growth rotation. Mandibular rotations are more pronounced than maxillary rotations (Björk and Skieller, 1977) and were first described by Björk (1955) with the aid of metallic implants. Björk (1969) described three centres of rotation of the mandible; the anterior, posterior or middle parts of the mandible (Figure 1.4). The reason why these rotations vary from one individual to another is not clearly understood. However, growth of the condyle (Dibbets, 1990), the lower border of the mandible (Björk, 1969) and the cranial base (Björk and Skieller, 1983) all contribute to mandibular growth rotations.

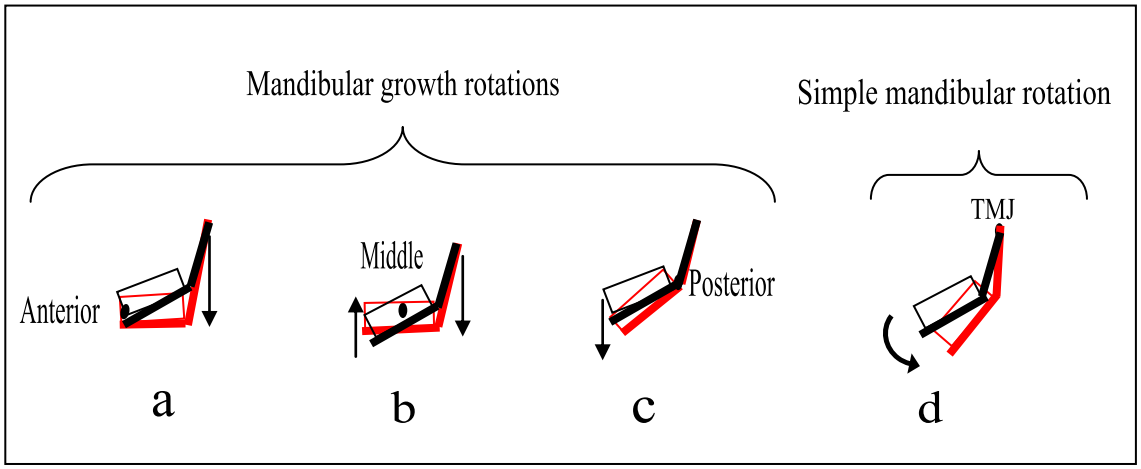


Figure 1.4: Mandibular growth rotations. a) *If the centre of rotation is located at the lower anterior part of the mandible (incisor area), the mandible will move in a forward direction with an increased posterior face height and a normal growth of the lower anterior face height.* ***b)*** *An inverse relationship between the anterior and posterior face heights (increased posterior and reduced anterior face heights and vice versa) would occur if the centre of rotation is at the midpoint of the mandible (premolar region).* ***c)*** *If the posterior part of the mandible (the most distal lower molar area) is the centre of rotation, the mandible will rotate backwards with an increase in the lower anterior face height.* ***d)*** *When the mandible achieves a pendulum movement and swings either forwards or backwards around the TMJ this is considered a simple mandibular rotation rather than a growth rotation. This can be seen in patients with muscular dystrophy, where the mandible simply rotates backwards as a result of weak muscles, or during orthodontic treatment which causes extrusion of the molar teeth. TMJ: Temporo-mandibular joint.*

Variations in the growth or development of craniofacial skeletal or muscular tissues, during intrauterine or postnatal periods, may cause differences in craniofacial features. These differences could be within what is considered a normal range, as seen between individuals, or could be extreme as seen in patients with craniofacial deformities.

1.4. CRANIOFACIAL DEFORMITIES

Craniofacial deformity is a broad term that includes all anomalies associated with any of the skull bones (Sperber, 1989). The UK National Statistics Office (2007) reported 683 live births with congenital craniofacial anomalies. The majority were cleft patients (324 live births), followed by syndromic patients with craniofacial deformities as a result of chromosomal abnormalities (320 live births), craniosynostotic conditions (28 live births) and the least were syndromic patients with craniofacial malformations that have developed due to teratogenic factors (11 live births). Nevertheless, other craniofacial developmental deformities have been reported in non-syndromic individuals (Björk, 1950; Sassouni, 1969; Opdebeeck and Bell, 1978). As a result of the variable clinical phenotypes and different pathogenic mechanisms of craniofacial abnormalities, the Committee on Nomenclature and Classification of Craniofacial Anomalies of the American Cleft Palate Association proposed a simple classification of craniofacial anomalies based on aetiological factors, anatomical features and treatment need (Whitaker et al., 1981). This classification has grouped patients into five main categories: 1) Clefts; 2) Synostoses; 3) Atrophy (or hypoplasia); 4) Neoplasia (or hyperplasia); 5) Unclassified.

The unclassified craniofacial anomalies include either single or multiple organs and are best described by the shape of the affected part of the body (Whitaker et al., 1981). The organs are the tongue, nose, eyes, lips, ears and jaws. Single organ deformities are often seen in non-syndromic individuals while, if multiple organs are involved, the situation is mainly syndromic and can be seen as part of any other craniofacial anomalies. Although the mechanism of development of unclassified anomalies might be different between syndromic and non-syndromic individuals, the clinical phenotype is similar. Orthodontists are often confronted with jaw deformities seen in all three dimensions of both syndromic and non-syndromic individuals. An example of jaw deformity seen in syndromic patients is the retruded position of the maxilla observed in cleft patients

(Meazzini et al., 2008) or Crouzon syndrome (Wilkie, 1997), a prognathic mandible in Klinefelter syndrome (Gorlin et al, 1965), long face deformity seen in patients with muscular dystrophy (Kreiborg et al., 1978) and unilateral mandibular discrepancy reported in hemifacial microsomia (Poswillo, 1974). Figure 1.5, shows the similarity of clinical appearance of jaw deformities observed in syndromic patients compared to non-syndromic individuals.

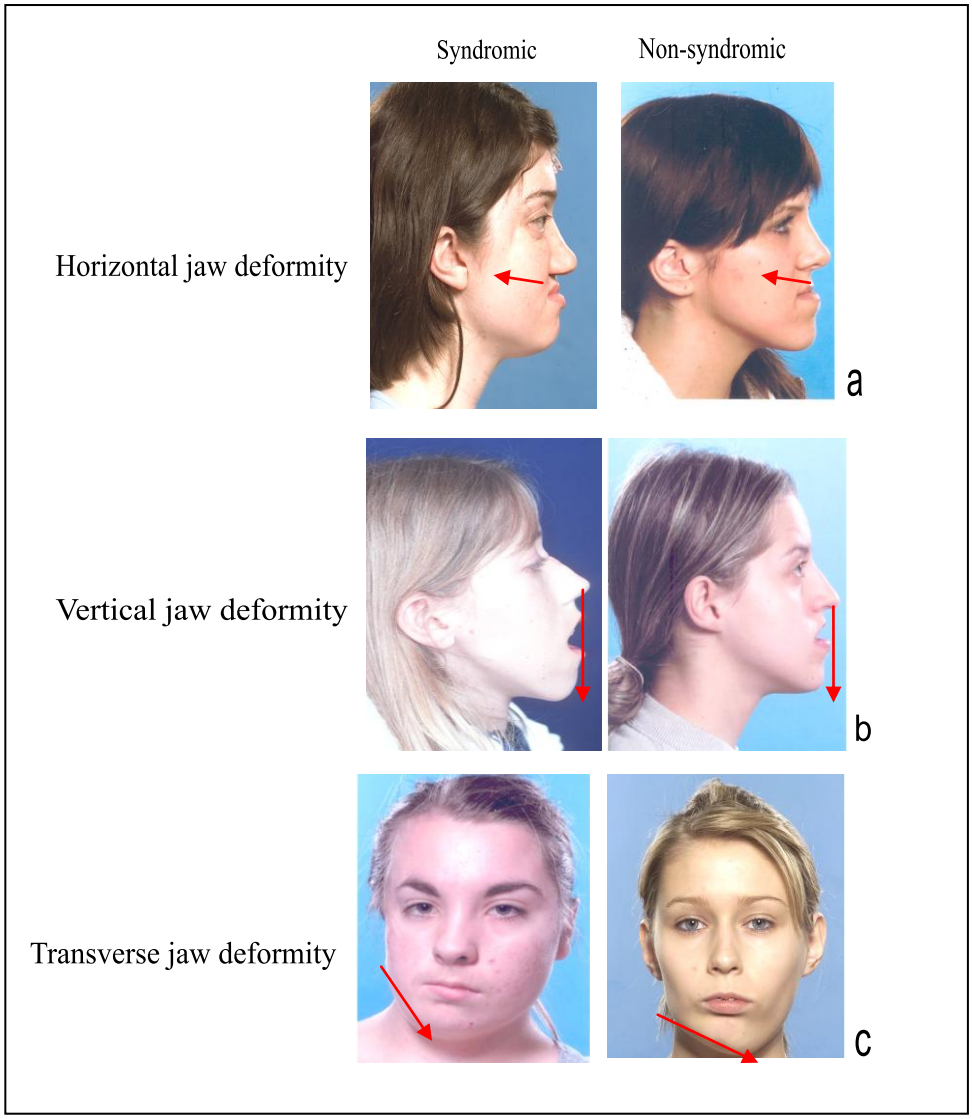


Figure 1.5: Unclassified jaw deformities seen in all three dimensions of both syndromic and non-syndromic individuals. a) *A female patient with Crouzon syndrome (fibroblast growth factor receptor-FGFR- gene mutation) having a prognathic lower jaw appearance as a result of an under developed maxilla, while the non-syndromic female has no craniosynostoses or any other deformities and is showing similar clinical jaw appearance.* ***b)*** *Two patients with a similar long face clinical appearance. The syndromic patient has muscular dystrophy, while the non-syndromic female has abnormal vertical facial growth with no muscular disease.* ***c)*** *A syndromic female with hemifacial microsomia compared to a non-syndromic female with similar unilateral mandibular discrepancy as a result of hemimandibular hyperplasia.*

1.4.1. DESCRIPTION OF JAW DEFORMITIES

Jaw deformities are described clinically based on the vertical, horizontal and transverse position of the mandible in relation to the maxilla (Björk, 1955). Patients with transverse skeletal problems have been reported with asymmetric masseter muscle structure (Kwon et al., 2007) and function (Dong et al., 2008), which may subsequently result in various transcriptome levels, depending on the investigated side of the masseter muscle. This aspect is beyond the scope of this project. Therefore, only vertical and horizontal jaw deformities will be discussed further.

Jaw deformities often have both dental and skeletal components including, the maxilla, the mandible and both upper and lower dentitions. For diagnostic purposes and to ease treatment planning, orthodontists have combined both dental and skeletal components and have identified four basic facial patterns (Sassouni, 1969). Two facial patterns were reported in the vertical dimension, the long (Schendel et al., 1976) and short (Opdebeeck and Bell, 1978) faces, and two other extremes have been identified in the horizontal dimension, namely Class II and Class III (Houston et al., 1992). These patterns were applied during the assessment of both syndromic and non-syndromic individuals regardless of the aetiological factors that have resulted in the development of these features.

1.4.2. BASIC CRANIOFACIAL PATTERNS

1.4.2.1. Vertical facial patterns

Orthodontists usually assess vertical facial development by measuring the lower anterior face height (LAFH) which extends from under the nose to the lowest point of the chin (Houston et al., 1992). The LAFH is compared to general facial proportions, and in average cases is almost equal to the mid and upper anterior face heights. This simple method is often used clinically to classify patients into average, long or short faces. Other dental and radiographic features are also used to confirm the clinical diagnosis. These are listed below.

1.4.2.1.1. The long face

Generally, patients with a long face pattern exhibit increased clinical vertical dimensions, including lower and total anterior face heights and excessive display of the upper anterior teeth, which can be seen with or without dental anterior open bite (AOB). Radiographic findings include increased total and lower anterior face heights, vertical maxillary excess and an increased mandibular gonial angle (Schendel et al., 1976; Fields et al., 1984) (Figure 1.6).

1.4.2.1.2. The short face

Short face individuals have clinically reduced vertical lower and total anterior face heights, dental deep bite, and a markedly reduced mandibular gonial angle as seen on radiographs (Opdebeeck and Bell, 1978) (Figure 1.6).

1.4.2.2. Horizontal facial patterns

Horizontal facial patterns are assessed by the antero-posterior (AP) position of the mandible in relation to the maxilla and the cranial base (Björk, 1950). A patient is diagnosed clinically as having an average antero-posterior profile when the position of the mandible is at or slightly behind the maxilla (Houston et al., 1992). Two main groups are associated with horizontal patterns, the Class II and Class III.

1.4.2.2.1. Class II

Skeletal Class II is described clinically by the position of the mandible posterior to the maxilla with a retrognathic – bird-beak like appearance (Björk, 1955). Similarly, the dental appearance is characterised by a distal position of either the lower incisors (Foster and Day, 1974) or the lower first molars (Ackerman and Proffit, 1969) in relation to the corresponding upper dentition. The radiographic appearance of a Class II pattern is often characterised by features such as a long anterior cranial base (Hopkin et al., 1968) a short posterior cranial base (Sayin and Turkkahraman, 2005), an obtuse cranial base angle (Enlow et al., 1982), a protruded position of the maxilla in relation to the cranial base (Ishii et al., 2002), increased maxillary length (Dhopatkar et al., 2002) and short mandibular body length (Enlow et al., 1982) (Figure 1.6).

1.4.2.2.2. Class III

Skeletal Class III is described clinically by forward positioning of the mandible ahead of the maxilla to give a prognathic appearance. Dentally, the lower incisors (Foster and Day, 1974) or the lower first molars (Ackerman and Proffit, 1969) are in a mesial position in relation to the corresponding upper dentition. Radiographically, Class III patients are often characterised by a short anterior cranial base (Björk, 1950; Hopkin et al., 1968; Ishii et al., 2002), a large posterior cranial base (Ngan et al., 1997) and an acute cranial base angle (Battagel, 1993; Proff et al., 2008). The maxilla may be short in horizontal length (Guyer et al., 1986) while the mandibular body length may be significantly increased (Sanborn, 1955, Dhopatkar et al., 2002) (Figure 1.6).

Patient classification according to the various facial patterns is further complicated for two reasons. First, the long face (Schendel et al., 1976), short face (Opdebeeck and Bell, 1978), Class II (Antonini et al., 2005) and Class III (Jacobson et al., 1974) have all been reported with various subdivisions and varying degrees of maxillary, mandibular and dental discrepancies. Second, it is common to see patients with a combination of both vertical and horizontal patterns rather than a single dimensional discrepancy (e.g. Class III long face or a Class II short face patient) (Sassouni, 1969; Proffit and White, 1990). The difficulties associated wih patient classification for genetic research (phenotype-genotype relationship) will be discussed in Chapter 5. Figure 1.6 shows the classical appearance of the four basic facial patterns compared to an average individual.

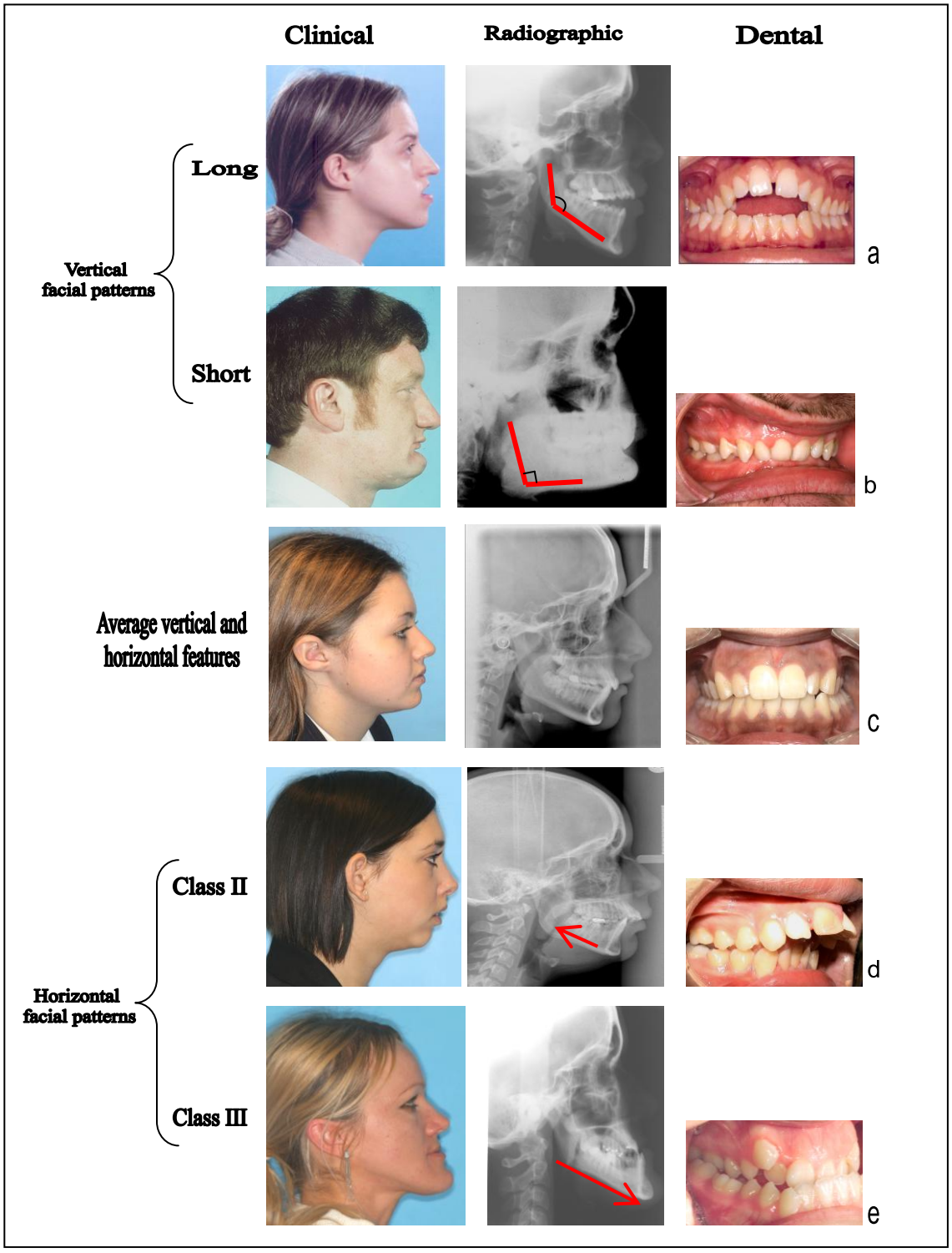


Figure 1.6: The classical clinical, dental and radiographic appearance of the various facial patterns. *All patients were non-syndromic.* ***a)*** *Clinical long face pattern showing radiographic obtuse mandibular gonial angle and a dental AOB.* ***b)*** *Short face pattern with reduced mandibular gonial angle and a dental deep bite.* ***c)*** *A patient with average vertical and horizontal clinical, radiographic and dental features.* ***d)*** *A clinical Class II pattern with a retruded position of both the mandible and the lower dentition.* ***e)*** *A Class III pattern with a protruded appearance of both the mandible and the lower dentition.*

1.4.3. PREVALENCE OF CRANIOFACIAL PATTERNS

The prevalence of different facial patterns varies between different populations (Altemus, 1959; Foster and Day, 1974; Soh et al., 2005; Hassan, 2006). However, most of these epidemiological studies were based on dental patterns rather than skeletal components of non-syndromic individuals (Table 1.2).

Table 1.2: Vertical and horizontal dento-facial patterns in various populations.

Population type	n	Age range (Years)	Horizontal patterns		Vertical patterns	
			Class II%	Class III%	DB %	AOB %
Asians (Soh et al., 2005)						
Chinese	258	17-22	26	23	11	5
Malaysian	60	17-22	18	27	13	2
Indians	21	17-22	57	5	5	0
Africans						
Nigerians (Onyeaso, 2004)	334	12-17	14	12	9	14
Kenyans (Ng'ang'a et al., 1996)	919	13-15	10	0	7	8
African Americans (Altemus, 1959)	3500	12-16	11	5	*	*
Caucasians						
British Caucasian (Foster and Day, 1974)	1000	11-12	27	3	54	2
Saudi Arabian Caucasian (Hassan, 2006)	743	17-24	34	4	34	20

n: Number of subjects included in the study. DB: Deep bite. AOB: Anterior open bite. *Not reported.

Although the Class II and deep overbite patterns have a general higher prevalence than the Class III and AOB malocclusion in most populations, the Class III and AOB cases were more likely to seek surgical correction of their deformities later in life (Al-Deaij, 2001; Chew, 2006) with females having a generally higher ratio than males, almost 2:1 (Bailey et al., 2001; Ong, 2004). This was mainly attributed to the aesthetic (Ong, 2004) and functional (speech and chewing) problems associated with Class III and open bite cases (Rivera et al., 2000).

1.4.4. AETIOLOGY OF CRANIOFACIAL PATTERNS

As stated earlier, vertical and horizontal jaw deformities can be seen in both syndromic and non-syndromic individuals. However, the mechanism of development of such anomalies in syndromic patients has been mainly attributed to variations and mutations on a genomic level, such as T-box gene mutations in cleft patients (Arnold et al., 2006) or fibroblast growth factor receptor (FGFR) gene mutations, seen in craniosynostotic conditions. In contrast, observational genetic studies of non-syndromic individuals, with developmental jaw deformities have revealed a multifactorial type of inheritance (Litton et al., 1970) and varying degrees of effect of both genetic and environmental factors (Hunter, 1965; Dudas and Sassouni, 1973; Nakata et al., 1973). This may indicate the need for a more complex approach to evaluate the genetic predispositions of developmental craniofacial patterns seen in non-syndromic individuals than for syndromic patients. This is due to the fact that the transcriptome expression of non-syndromic individuals is modulated by both genetic and environmental factors, which makes it difficult to relate a specific gene to the clinical morphology. This suggests the importance of considering both genomic and transcriptome backgrounds during the assessment of non-syndromic individuals with various craniofacial patterns.

1.4.4.1. Genetic studies of non-syndromic craniofacial patterns

Genetic studies aimed at observing familial inheritance of non-syndromic craniofacial patterns between parents and offspring (Hunter et al., 1970; Johannsdottir et al., 2005), siblings (Harris and Johnson, 1991) and twins (Hunter, 1965; Carels et al., 2001), have revealed that linear features, such as mandibular body length (Carels et al., 2001; Johannsdottir et al., 2005), lower anterior (Dudas and Sassouni, 1973) and posterior face heights (Johannsdottir et al., 2005) were all under a strong genetic influence. While upper anterior face height has been found to be influenced by genetic factors only during the early stages of development, later in life it has largely responded to environmental demands (Dudas and Sassouni, 1973). Angular features on the other hand, such as gonial angle, Sella-Nasion-A-point (SNA) and Sella-Nasion-B-point (SNB) are largely controlled by environmental factors (Carels et al., 2001).

The use of familial information is considered a valuable tool to ascertain the features inherited from other craniofacial parameters which can respond to environmental

stimulus (Harris and Kowalski, 1976; Carels et al., 2001). This would help clinicians to innovate appliances or customise treatment to take advantage of the surrounding environment and can contribute positively to the remodelling response and thereby help to stabilise the results of treatment.

1.4.4.2. Environmental factors affecting craniofacial patterns

Several environmental factors, for example, thumb sucking (Heimer et al., 2008), respiratory need (Linder-Aronson, 1979), mouth breathing (Faria et al., 2002), muscular dystrophy (Eckardt and Harzer, 1996) and obesity (Sadeghianrizi et al, 2005) have been associated with specific craniofacial patterns. However, the mechanical load exerted by the masticatory muscles (Kiliaridis, 1995) in response to normal physiologic movements (Kubota et al., 1998), pathologic conditions (Matic et al., 2007), type of diet (Helm and German, 1996), orthodontic appliances (Kuster and Ingervall, 1992) and exercise (English and Olfert, 2005) have also been considered major environmental stimuli that may alter craniofacial morphology (Gedrange and Harzer, 2004).

Furthermore, masseter muscle activity (Kobayashi et al., 2001), size (Satiroğlu et al., 2005), volume (Kitai et al., 2002), thickness (Kiliaridis and Kalebo, 1991), cross-sectional area (Kitai et al., 2002), direction and orientation of the muscle fibres (Ariji et al., 2000) and masticatory efficiency (Kim and Oh, 1997) have been found to be significantly different between patients with variable craniofacial patterns.

1.5. MASSETER MUSCLE STRUCTURE AND FUNCTION IN RELATION TO CRANIOFACIAL PATTERNS

1.5.1. MASSETER MUSCLE ACTIVITY

Muscular activity can be recorded using electromyography (EMG). Using this method it has been shown that muscle activity in Class III long face subjects is lower than in subjects with Class I average and long faces, Class III average faces and Class II individuals with average and long faces (Cha et al., 2007). Masseter muscle activity in relation to pure vertical features has been reported as being lowest in long face individuals (Lowe, 1980) and highest in short face patients (Ueda et al., 2000; Serrao et al., 2003) compared to controls.

1.5.2. MASSETER MUSCLE SIZE AND SHAPE

The size, volume, thickness, cross-sectional area and the direction and orientation of the muscle fibres have been measured using different types of magnetic resonance imaging (MRI) (van Spronsen et al., 1992), computerized tomography (CT scan) (Kitai et al., 2002), and ultrasonography (Kiliaridis and Kalebo, 1991). The masseter muscle has been reported as short, thin, of low volume and having a small cross sectional area in Class III (Ariji et al., 2000; Kitai et al., 2002) and long face patients (Kiliaridis and Kalebo, 1991; van Spronsen et al., 1992) when compared to controls. However, when compared to other facial patterns, long face individuals showed the thinnest, while short face patients exhibited the thickest masseter muscle volume compared to average face subjects (Satiroğlu et al., 2005). Furthermore, the orientation of the masseter muscle fibres in Class III patients compared to the controls was found to be in a more forward direction, forming an obtuse angle with the Frankfort horizontal plane (Ariji et al., 2000; Kitai et al., 2002). It has been suggested that the more upright the direction of the masseter muscle fibres (as in short face patients) in relation to the Frankfort horizontal or functional occlusal planes, the greater the occlusal forces (Kitai et al., 2002).

Other studies have assessed the relationship between the volume of the masseter muscle and specific craniofacial skeletal parameters. The results have indicated a positive correlation between masseter muscle volume and the ramus height (Kubota et al., 1998), posterior face height (Benington et al., 1999) and the cross-sectional area of the zygomatic arch (Kitai et al., 2002), while a negative correlation was observed in relation to mandibular inclination and gonial angle (Kubota et al., 1998; Benington et al., 1999). No relationship was found between masseter muscle volume and cranial width (Kitai et al., 2002). Furthermore, general anterior and posterior craniofacial vertical dimensions were more related to masseter muscle volume than cross-sectional area (Boom et al., 2008).

1.5.3. MASSETER MUSCLE EFFICIENCY

Masticatory muscle efficiency has been defined as the capability of the muscles to produce different functional commands such as swallowing, simultaneous chewing, and maximum occlusal bite (Gambareli et al., 2007). These commands can be affected by several factors such as the number of teeth acting, as well as the bite forces generated by

the muscles of mastication. Therefore, masseter muscle efficiency has been assessed mainly via recording occlusal bite forces (Hunt and Cunningham, 1997) and counting the number of occlusal contacts (Shiratsuchi et al., 1991). Both long face individuals (Ingervall and Helkimo, 1978; Proffit et al., 1983) and Class III patients (Ellis et al., 1996; Iwase et al., 2006) have demonstrated the lowest number of occlusal contacts and reduced bite forces when compared to average, short face and Class II subjects.

1.5.4. MASSETER MUSCLE FIBRE TYPE COMPOSITION

Patients with short faces and dental deep bites have been reported as possessing higher numbers of fast contracting than slow contracting muscle fibres (Rowlerson et al., 2005; Hunt et al., 2006). On the other hand, both long face and average vertical dimension patients had higher proportions of slow contracting than fast contracting fibres, with the controls having a higher percentage than long face subjects. These findings were 50% and 43%, respectively (Boyd et al., 1989). Class II and Class III patients on the other hand, had a similar fibre type distribution (Rowlerson et al., 2005) with the number of slow contracting fibres being greater than that of fast contracting fibres. Nevertheless, Class III subjects had a large presence of hybrid fibres, particularly on the non-chewing side when compared to the Class I controls (Ringqvist, 1974). This was also evident in long face patients where hybrid fibres composed 43% of the fibre types compared to short face patients with only 9% (Rowlerson et al., 2005).

The relationship between craniofacial form and muscle structure has been further investigated based on the MyHC protein content. Neonatal MyHC that is usually abundant in the hybrid fibres has been found to be inversely related to the number of occlusal contacts (i.e. the lower the number of occlusal contacts the higher the expression of the MyHCneo protein) (Nelson-Moon et al., 1998; Hunt et al., 2006).

The question remains as to whether the reported muscle differences between patients with variable craniofacial phenotypes were due to pre-programmed genetic defects of masseter muscle composition and hence function or due to adaptive behaviour by the masticatory musculature in response to abnormally-developing skeletal structures. Further investigations have, therefore, been undertaken to understand musculo-skeletal function in patients with different craniofacial patterns.

1.6. EXPERIMENTAL STUDIES OF MUSCULO-SKELETAL FUNCTION

1.6.1. THE EFFECT OF DIET

Animal models have been used to assess the effect of the physical properties of diet on the development of craniofacial phenotypes via the mechanical load exerted by the masseter muscle. Pigs (Helm and German, 1996) and rats (Kiliaridis et al., 1985) that were fed a soft diet had less masseter muscle activity and increased vertical facial development, but reduced growth rates of the cranial base dimensions and bone deposition at the gonial region and the lower border of the mandible, compared to the controls who were fed a normal diet. Further investigation was recommended to establish the effect of diet composition on muscular activity and bone remodelling (Kiliaridis et al., 1985).

1.6.2. THE EFFECT OF EXERCISE

Masticatory muscle exercise has been found to increase the functional capabilities of the masseter muscle (Kiliaridis, 1995). However, very few researchers have investigated the recruitment of the mechanical and physical properties of the masseter muscle produced by exercise as a supporting treatment for different craniofacial deformities, particularly patients with anterior open bite (AOB). English and Olfert (2005) demonstrated that masseter muscle exercise could be used synergistically with other orthodontic appliances to treat skeletal AOB without the need for orthognathic surgical correction. However, extensive research is still required to explain musculo-skeletal adaptability to exercise (Hamilton and Booth, 2000).

1.6.3. THE EFFECT OF ORTHODONTIC APPLIANCES

The mechanism of action of posterior bite blocks on the treatment of patients with AOB has been explained in terms of intrusion of the posterior teeth, allowing a closing autorotation of the mandible and an upright direction of the masseter muscle fibres in relation to the occlusal plane, which subsequently increases the occlusal bite forces and masseter muscle activity (Kuster and Ingervall, 1992). This mechanism suggests that the masseter muscle adapts to modified skeletal and dental morphology. In contrast, the results of Bresin and Kiliaridis (2002) have explained the action of bite blocks in a reverse manner. This later study included two groups of young rats, one of which has

been fed a hard diet while the other was fed a soft diet; the aim being to generate masseter muscles with different functional capabilities. Both groups were then supplied with posterior bite blocks. The rats with the soft diet had weaker muscle activity which in turn affected the intrusion capacity of the bite blocks compared to the hard diet group. This work suggests that masseter muscle efficiency affects the capabilities of bite blocks and further adaptability of skeletal structures.

1.6.4. THE EFFECT OF ORTHOGNATHIC SURGICAL TREATMENT

The process of orthognathic surgery requires the integration of pre-surgical orthodontics, surgery and post-surgical orthodontic phases (Proffit and Fields, 2000). The masseter muscle undergoes a functional (Hunt and Cunningham, 1997) and structural (Boyd et al., 1989) adaptation in response to the different functional demands during the three stages of orthognathic treatment (Kobayashi et al., 2001). Table 1.3 summarises these differences.

Table 1.3: The effect of the three stages of orthognathic treatment on the structure and function of the masseter muscle.

Orthognathic treatment stage	Effect on the masseter muscle	Proposed reason
Pre-surgical orthodontic stage (Kobayashi et al., 2001)	-Increased muscle activity.	-Aligned teeth.
Surgical stage* (Ellis et al., 1996) (Mayo et al., 1988)	-Reduced bite force. -General muscle atrophy. -Reduction in CSA of fast and slow fibres in bimaxillary cases.	-Patients cautious about the bite.
Post-surgical orthodontic stage Short-term (≥ 1 year) (Hunt and Cunningham, 1997) (Kim and Oh, 1997) Long-term (1 year <) (Iwase et al., 2006)	-Reduced bite force in Class II. -Increased bite force in Class III. -Reduction in slow fibres. -Increase in fast fibres. -General rise in muscle efficiency but not to the level of patients with average facial features.	-Aligned teeth and jaws and improved muscle activity. -Reduced regenerative abilities of masseter muscle.

* Immediately after surgery and up to 6 months following surgery. CSA: Cross sectional area.

This has raised the question as to whether the molecular composition of the masseter muscle could be considered as a marker for a specific facial pattern or as a predictor for the success of treatment. This has led to the investigation of the masseter muscle on a gene expression level.

1.7. GENE EXPRESSION STUDIES OF THE MASSETER MUSCLE

Due to the lack of knowledge of the full genetic background of the masseter muscle it has been difficult to propose a set of candidate genes to be tested in response to masseter muscle stimulation. However, scientists have selected candidate genes as follows:

- Genes which encode previously identified contractile and unique MyHC proteins of the masseter muscle (*MYH* genes) (Ringqvist et al., 1982; d'Albis et al., 1993; Smerdu et al., 1994).

- Genes which encode some of the modulatory proteins which have been reported to have a major role in masseter muscle regeneration (such as fibronectin) (Price et al., 1998), and remodelling (such as matrix metalloprotenases – MMPs) (Singh et al., 2000).

- Genes which have been reported in regenerating muscles in response to mechanical stimulus (such as insulin-like growth factors (IGF)) (Goldspink, 2006; Maricic et al., 2008).

- Myostatin gene which has been suggested to be involved in the adaptive behaviour of the masseter muscle (Maricic et al., 2008).

Investigation of masseter muscle genes in patients with average and extreme craniofacial patterns has only recently been undertaken, and therefore very few studies are cited in the literature. Table 1.4 summarises the main findings of studies related to genes of the masseter muscle and their expression status in relation to various craniofacial patterns, and in response to different treatment stimuli.

Table 1.4: Studies of masseter muscle genes in relation to craniofacial patterns and in response to orthognathic surgery.

Gene symbol	Main study findings
*MYH*1 (fast IId/x)	
(Suchak et al., 2009)	-Inverse correlation to MMa
(Gedrange et al., 2005)	-Low amounts in both Class II and Class III
(Nelson-Moon et al., 1998; Suchak et al., 2009)	-No differences in long face vs. Ctrl
*MYH*2 (fast IIa)	
(Gedrange et al., 2006)	-Higher in Class II than Class III patients
(Maricic et al., 2008)	-Increased following surgery (in both Class II and III)
(Harzer et al., 2007)	-Positive correlation to the DOC
(Nelson-Moon et al., 1998; Suchak et al., 2009)	-No differences in long face vs. Ctrl
*MYH*3 (embryonic)	
(Nelson-Moon et al., 1998)	-Inverse correlation to the DOC
(Nelson-Moon et al., 1998; Suchak et al., 2009)	-No differences in long face vs. Ctrl
*MYH*6 (α –cardiac)	
(Nelson-Moon et al., 1998; Suchak et al., 2009)	-No differences in long face vs. Ctrl
*MYH*7 (slow I)	
(Gedrange et al., 2006)	-Higher in Class II than Class III patients
(Maricic et al., 2008)	-Lower expression following surgery
(Harzer et al., 2007)	-Inverse correlation to the DOC
(Nelson-Moon et al., 1998; Suchak et al., 2009)	-No differences in long face vs. Ctrl
*MYH*8 (neonatal)	
(Nelson-Moon et al., 1998; Suchak et al., 2009)	-No differences in long face vs. Ctrl
Fibronectin*	
(Price et al., 1998)	-No differences in long face vs. Ctrl
MMP1, MMP2, MMP9, TIMP2	
(Singh et al., 2000)	-No differences in long face vs. Ctrl
Myostatin	
(Maricic et al., 2008)	-No differences before and after surgery
(Maricic et al., 2008)	-Reduced expression in females than males
IGF**	
(Maricic et al., 2008)	-Increased after surgery up to 6 months

MMa: Maxillary-mandibular angle. Ctrl: Control. DOC: Dental occlusal contacts. *MYH*: Myosin heavy chain genes. MMP: Matrix metalloproteinases. TIMP: Tissue inhibitor metalloproteinases. *Embryonic fibronectin splice variant EIIIA. **Insulin-like growth factor I-splice variant Ec (IGF-IEc).

It is evident from the previous review that the masseter muscle holds unravelled potentials in terms of craniofacial development as well as its adaptive behaviour to various treatment modalities. However, the genetic explanation of such potentials is still in its infancy, as only small numbers of human masseter muscle genes have been investigated and no reliable predictors have been found to date. This may have been partly related to the limitations of molecular methods previously available. However, with the development of new technologies, it may be possible to discover new candidate genes which might take part in postnatal musculo-skeletal development and adaptation.

1.8. THE SCOPE OF GENE EXPRESSION TECHNOLOGIES

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) is one of the most sensitive and reliable techniques that has been developed to measure the abundance of either DNA or RNA (VanGuilder et al., 2008) and has been used in both human (Suchak et al., 2009) and animal (Ödman et al., 2008) masseter muscle gene expression research. However, qRT-PCR provides low-throughput data, whereby only a few genes can be tested (Moody, 2001). Microarray on the other hand, is a high-throughput type of gene expression technique where thousands of genes can be tested in one experiment. New genes have been found using this technique (VanGuilder et al., 2008). Gene expression microarrays have been used to reveal part of the complicated molecular pathway in relation to various craniofacial anomalies.

1.8.1. GENE EXPRESSION MICROARRAYS IN OROFACIAL CLEFTS

Cleft lip and/or palate patients have been reported with various genetic mutations (Jezewski et al., 2003; Marcano et al., 2004; Andreou et al., 2007). However, it is not clear how these mutations function at a molecular level to develop syndromic and non-syndromic orofacial clefts (Stanier and Moore, 2004). Park and colleagues (2006) have conducted a large microarray genotypic study of several families with neonates who were identified with non-syndromic orofacial clefts and were further linked to gene expression-based network data. The study discovered new candidate genes in various embryonic tissues of cleft lip and palate patients which had not been previously identified. It has been suggested that investigating these genes on various molecular levels would provide a new insight into the development of both syndromic and non-syndromic orofacial clefts.

1.8.2. GENE EXPRESSION MICROARRAYS IN CRANIOSYNOSTOTIC CONDITIONS

Although both Crouzon and Apert syndromes have been reported with craniosynostoses (Wilkie, 1997) and fibroblast growth factor receptor 2 (FGFR2) gene mutations (Anderson et al., 1998), both syndromes have different clinical phenotypes based on cranial, facial and limb variations (Kimonis et al., 2007). The molecular genetic mechanism responsible for such differences has not been explained. Carinci and colleagues (2002) in a microarray gene expression experiment using fibroblast cells derived from the parietal bone of adult Crouzon and Apert syndrome patients during cranial surgery have found interesting results. Out of almost 19,000 cDNA (complementary DNA) sequences that were covered in the microarray experiment, two distinct sequences had differential expression between Crouzon and Apert patients. This discovery suggests different genetic mechanisms of the FGFR2 gene in the development of Crouzon and Apert phenotypes.

1.8.3. GENE EXPRESSION MICROARRAYS IN HEMIFACIAL MICROSOMIA

Hemifacial microsomia (HFM) is one of the most common syndromes associated with faulty development of the 1st and 2nd branchial arches with no familial predisposition (Poswillo, 1974). However, the genetic cascade arising from the faulty development of the branchial arches leading to the asymmetric facial appearance has not been revealed. Cai and co-workers (2005) have studied extensively the gene expression profile of the 1st branchial arch in various craniofacial structures of aborted embryos using different high throughput gene expression techniques and comparing it to mouse models. The study has identified novel genes which were not reported earlier in the 1st branchial arch of humans. Furthermore, thousands of genes were expressed differently in the 1st branchial arch between the 4th and the 5th week of embryonic development and were suggested to be candidate genes related to craniofacial anomalies such as orofacial clefts and hemifacial microsomia.

1.9. STATEMENT OF THE PROBLEM

Variations in the structural (Boyd et al., 1989; Rowlerson et al., 2005) and functional (Hunt and Cunningham, 1997; Ariji et al., 2000) properties of the masseter muscle between non-syndromic patients with various craniofacial patterns are well documented. However, the genetic explanation of these differences is still pending and has yet to be revealed. No previous gene expression experiment has incorporated microarray technology to identify potential masseter muscle candidate genes in relation to various non-syndromic craniofacial patterns. Furthermore, the effect of the different vertical and horizontal craniofacial deformities, dental occlusion and cephalometric parameters on the gene expression of the myosin heavy chain genes of the masseter muscle has not been established fully.

The current project is part of a large international research study conducted in collaboration with UCL/ Eastman Dental Hospital (EDH) in the United Kingdom and Riyadh Military Hospital (RMH) in the Kingdom of Saudi Arabia (KSA). DNA genotyping and RNA gene expression microarrays are used to assess both the masseter muscle and the bones of the face of patients with variable craniofacial patterns before, during and after orthognathic surgery. However, the current study is included to provide the foundations for the main research to progress and is designed to identify potential novel masseter muscle candidate genes to be analysed and tested further in relation to craniofacial deformities.

1.10. AIMS OF THE PROJECT

The aims of the current study are to use gene expression microarray technology to ascertain whether any novel masseter muscle genes can be identified which respond differently in relation to various craniofacial patterns.

The null hypotheses for this research are:

1. No novel masseter muscle genes can be identified in relation to patients exhibiting different craniofacial patterns when using microarray technology.

2. There is no relationship between masseter muscle gene expression and various combinations of vertical and horizontal craniofacial patterns.

3. There is no correlation between masseter muscle gene expression and various vertical and horizontal cephalometric variables.

4. There is no correlation between masseter muscle gene expression and the number of dental occlusal contacts associated with craniofacial patterns.

1.11. LAYOUT OF THE THESIS

Chapter 1	Background -Review of the literature. -Identification of the problem and aims of the project.
Chapter 2	General materials and methods -Ethical and clinical design. -Gene expression techniques used and experimental design.
Chapter 3	Optimisation of total RNA extraction protocol -Standardization of RNA extraction technique. -Quality control of samples included in the research.
Chapter 4	Microarray gene expression of the masseter muscle -Quality control of microarray data. -Masseter muscle candidate gene lists and identification of novel genes.
Chapter 5	Difficulties of patient classification for genotype-phenotype studies -Identification of the different craniofacial subgroups of the subjects. -Regrouping of subjects based on various classifications.
Chapter 6	Masseter muscle genotype in relation to various craniofacial phenotypes -Gene expression of novel and *MYH* genes in relation to various classifications. -Gene expression of novel and *MYH* genes in relation to radiographic parameters. -Gene expression of novel and *MYH* genes in relation to dental occlusal contacts.
Chapter 7	General discussion -Summary of the results -Masseter muscle genotype-phenotype relationship. -Conclusions and directions for future work.

Chapter 2. General materials and methods

2.1. INTRODUCTION

Several ethical, clinical and experimental issues were taken into account when designing the current study. This is due to the nature of the tissue samples required as well as the number of different operating systems and analytical procedures available to describe both microarray and quantitative RT-PCR. The aims of this chapter are to give an overview of the materials and methods and of the genetic techniques used.

2.2. CLINICAL DESIGN

The clinical design for the current research has been initiated by three main steps:

1. Setting the inclusion and exclusion criteria required for subject recruitment.

2. Initial sample size estimation.

3. A pilot audit where the inclusion and exclusion criteria were implemented during the assessment of previous hospital notes of patients who have attended the orthodontic/orthognathic clinic at the Eastman Dental Hospital (EDH).

This is to assess whether sufficient numbers of patients matching the inclusion and exclusion criteria could be gathered from one hospital site as well as the assessment of the types of deformities encountered.

2.2.1. INCLUSION AND EXCLUSION CRITERIA

The current research is a case-control study where two main cohorts were required; the deformity and the controls

- Inclusion criteria

  Control group:

- Caucasian.
- Non-syndromic.
- Requiring the extraction of either/ both lower third molars (to aid in obtaining the muscle biopsy).

- Having average vertical and horizontal clinical facial appearance with no facial asymmetry and require orthodontic treatment to correct dental malocclusion (to justify obtaining lateral cephalometric radiographs).

Deformity group:

- Caucasian.
- Non-syndromic.
- Requiring the extraction of either/ both lower third molars.
- Having a single or a combination of vertical and horizontal facial deformities with no facial asymmetry and requiring orthognathic correction of jaw deformity.

- Exclusion criteria

Any patient who was diagnosed with any of the following criteria was excluded from the study:

- Syndromic.
- Racial categories other than Caucasian.
- Having asymmetric facial development.
- Did not require the extraction of either of the lower third molars.
- Previously undergone orthodontic treatment or surgical correction of facial deformity.
- Diagnosed with endocrine, connective tissue, muscle, autoimmune or bleeding disorders, bone disease or using prescribed drugs on regular basis.

2.2.2. INITIAL SAMPLE SIZE ESTIMATION

It is appreciated that microarray technology allows analysis of a large number of genes from samples of limited availability. However, in the absence of data from a pilot study, sample size information can be obtained from previous experiments (Stekel, 2003). Based on previously controlled *in-vitro* studies where the target p-value was 0.01 and the target log ratio was 2-fold, the number of the required arrays per group was 6, assuming normal distribution of gene expression. However, *in-vivo* studies contain two main sources of errors compared to one source of error in an *in-vitro* experiment. The first error is the biological error which is introduced from genotypic variations between

individuals, whilst the second error is technical and may arise as a result of variations in sample collection, handling or purification of the genetic material, change of reagents or during hybridization. The Bloomsbury Centre for Bioinformatics/ Department of Computer Science at UCL has provided a sample size estimate of 11 patients pre group (letter attached at Appendix A). This was conducted using data from previous studies, a target p-value of 0.05 and a target log ratio of 1.4-fold change.

2.2.3. THE EDH AUDIT

Using the pre-determined inclusion and exclusion criteria of the deformity group and an estimated sample size per group, the hospital notes of all patients who were referred to the Eastman Dental Hospital (EDH) for surgical jaw correction in year 2004 were investigated. Surgical procedures were performed on 80 patients for the correction of facial deformities: 29 males and 51 females with an age range of 16-50 years, all non-syndromic except for 3 syndromic females and 9 non-specific cases such as trauma or distraction osteogenesis. This resulted in a total of 68 non-syndromic patients with various vertical and horizontal craniofacial patterns (Table 2.1).

Table 2.1: Craniofacial deformities included in the 2004 EDH audit.

Horizontal and vertical patterns	Number of patients
Class III long face without AOB	18 (including 3 asymmetry)
Class III average vertical face	11
Class III long face with AOB	5
Class III short face	3 (including 1 asymmetry)
Total	37
Class II long face without AOB	11
Class II long face with AOB	9
Class II short face	6
Class II average vertical face	5 (including 1 asymmetry)
Total	31

AOB: Anterior open bite.

However, of the 68 non- syndromic craniofacial deformities, only 17 patients with various craniofacial patterns matched the required inclusion and exclusion criteria. Furthermore, other ongoing projects at the EDH were recruiting individuals with similar criteria to the current research and, ethically, it was not possible to recruite the same patient for two different studies. These factors suggested that one hospital site may not be sufficient to gather the appropriate sample size of patients with the recommended inclusion and exclusion criteria. Therefore, other hospital sites were included.

2.3. ETHICAL APPROVAL

This study received three ethical approvals, one broad approval from the UCL Joint Research and Ethics Committees (REC) to be conducted at the Eastman Dental Hospital (EDH), as well as two other local ethical approvals to be conducted at associated sites including Whipps Cross University Hospital (WCH) in the UK and the Riyadh Military Hospital (RMH) in Saudi Arabia (all ethical approvals are included in Appendix A).

2.4. RESEARCH SUBJECTS

2.4.1. THE RECRUITMENT PROCEDURE

Following the UCL REC guidelines, the invitation letter, leaflet and information sheet were supplied to both deformity and control groups. Each patient was given at least 24 hours to decide upon participation in the study (documents supplied to research participant is included in Appendix A).

2.4.2. RETROSPECTIVE RECALCULATION OF SAMPLE SIZE

Following the microarray experiment, we have used our own data in a special microarray sample size estimation website:

http://bioinformatics.mdanderson.org/MicroarraySampleSize/

This is to calculate retrospectively the sufficiency of the sample size included in the current study. The website required a set of criteria that were mainly obtained from the microarray experiment. The following section will list the criteria that were used for the current research to recalculate the sample size.

- Total number of genes (this information was obtained from the GeneChip® data sheet (Affymetrix®, 2003) = 38,500 genes.

- Acceptable number of false positive genes (1 is mainly a standard that is used in microarray experiments) = 1

- Desired fold change = 2

- Desired power (0.7-0.8 is a standard that is used in most microarray experiments) = 0.8

- Standard deviation (the SD was calculated from the gene expression intensities of all probes included on the microarray chip) = 0.36

Once all these criteria were submitted, an automatic computation of the sample size was obtained which revealed that 7 patients were recommended per group.

2.4.3. SUBJECTS INCLUDED

A total of 38 individuals were recruited from the Orthodontic departments of the EDH, WCH and RMH. Unfortunately, six deformity patients withdrew prior to biopsy collection leaving 32 patients for the study. The samples collected from the 32 patients were used in various experimental procedures such as standardisation of the RNA extraction protocol as well as the gene expression. This has resulted in a total of 29 patients included in the final gene expression analysis (Figure 2.1).

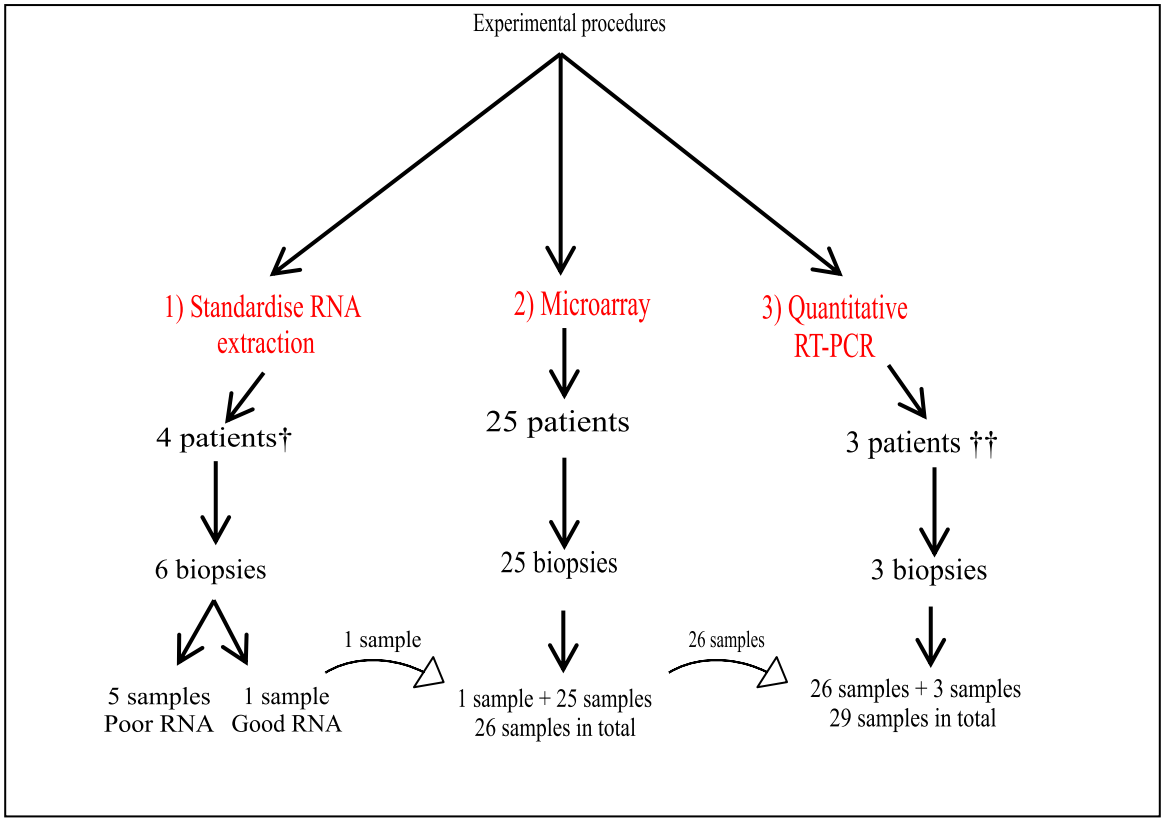


Figure 2.1: The number of patients and samples used in each experimental procedure. 1) *A total of 6 biopsies collected from 4 patients were used to optimise the RNA extraction protocol. One sample out of the 6 had good RNA quality and was included further in gene expression experiments.* ***2)*** *The sample with the good RNA quality as well as 25 other samples – each representing one patient, were used for gene expression microarray.* ***3)*** *The remaining aliquots of the total RNA of the 26 samples used in the microarray experiment as well as 3 other samples (total 29) were used for the quantitative RT-PCR.*

† Some patients had one biopsy while others had two biopsies; this was depending on whether one or both lower third molars were extracted. However, only one biopsy representing each patient was used for the experimental procedure except where indicated by †.

†† These patients were recruited at the initial stages of the study. However, due to patient related circumstances there was considerable delay in biopsy collection, and thus it was not possible to include these samples in the microarray analysis and were only included in the quantitative RT-PCR.

The 29 subjects consisted of 11 control females, with an age range of 16-34 years (mean age 22.66 $\pm$ 5 years) and 18 deformity patients (8 males and 10 females), with an age range of 16-36 years (mean age 23.94 $\pm$ 6 years). The general sample size of both the deformity and the control group was considered sufficient for the microarray experiment. However, the deformity group was further subdivided based on various clinical, dental and radiographic criteria, which resulted in a smaller sample size than

recommended in some of the deformity subgroups. The different subdivisions of the deformity group as relevant to the gene expression experiment will be discussed later.

2.4.4. LATERAL CEPHALOMETRIC RADIOGRAPHS

The lateral cephalometric radiographs were taken as part of the routine initial orthodontic diagnostic procedure of both the deformity and the control groups. Radiographs were taken at the three hospital sites using standardised cephalometric techniques. Each patient was oriented with the head in the natural head position and the teeth in centric occlusion.

2.4.4.1. Cephalometric landmarks

All pre-treatment lateral cephalometric radiographs of the subjects were hand-traced by the investigator using a sharp pencil, acetate paper and illuminated light box. Nine cephalometric landmarks were identified (Figure 2.2).

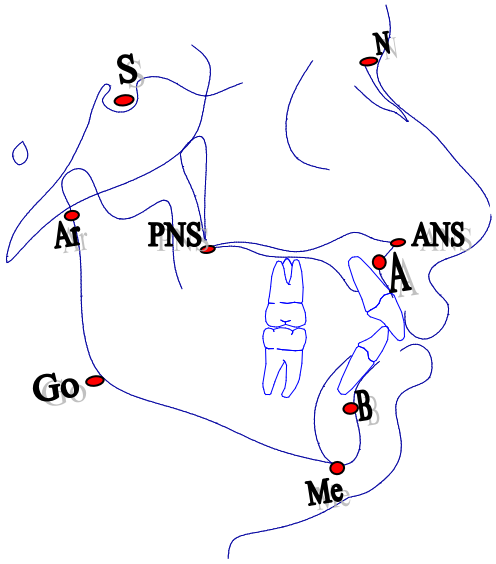


Figure 2.2: Cephalometric landmarks. *Sella (S), midpoint of sella turcica. Nasion (N), the most anterior point of the frontonasal suture. Articulare (Ar), the intersection between the posterior border of the mandible with the lower border of the posterior cranial base. Gonion (Go), the most posterior inferior point of the angle of the mandible. Menton (Me), the lowest point of the mandibular symphysis. Supramentale (B-point), the deepest point between the chin point and the mandibular alveolar crest. Subspinale (A-point), the deepest point between the anterior nasal spine and the maxillary alveolar crest. Anterior nasal spine (ANS), the tip of the bony process of the maxilla. Posterior nasal spine (PNS), the most posterior point of the bony hard palate in the sagital plane (Houston et al., 1992).*

2.4.4.2. Cephalometric variables

Based on the results of previous studies, the most significantly different vertical (Schendel et al., 1976; Opdebeeck and Bell, 1978) and horizontal (Björk, 1950; Reyes et al., 2006) cephalometric variables between average and craniofacial deformities were selected to be tested later against the gene expression data (Chapter 6).

- Horizontal variables

- ***SNA angle*** (sella-nasion-A point): Indicates the horizontal position of the maxilla in relation to the cranial base.
- ***SNB angle*** (sella-nasion-B point): Indicates the horizontal position of the mandible in relation to the cranial base.
- ***ANB angle*** (A point-nasion-B point): Indicates the relationship between the maxilla and the mandible in the horizontal direction.

- Vertical variables

- ***TAFH length*** (total anterior face height): A linear measurement from N to Me perpendicular to the maxillary plane (ANS-PNS).
- ***LAFH%*** (lower anterior face height percentage): The length of the LAFH (ANS-Me) perpendicular to the maxillary plane divided by the linear measurement of the TAFH (N-Me) and expressed as a percentage.
- ***TPFH length*** (total posterior face height): A linear measurement from S to Go perpendicular to ANS-PNS.
- ***RH length*** (Ramus height -Ar-Go): Linear measurement of the ramus of the mandible.
- ***SN-MP angle*** (SN-mandibular plane): Indicates the relationship of the mandibular plane (Go-Me) to the cranial base plane (S-N).
- ***SN-MxP angle*** (SN-maxillary plane): Indicates the relationship of the maxillary plane to the cranial base plane (S-N).

The SN-MxP was only used for the correction of the value of the ANB angle, as recommended by Mills (1987). Figure 2.3 demonstrates both horizontal and vertical measurements.

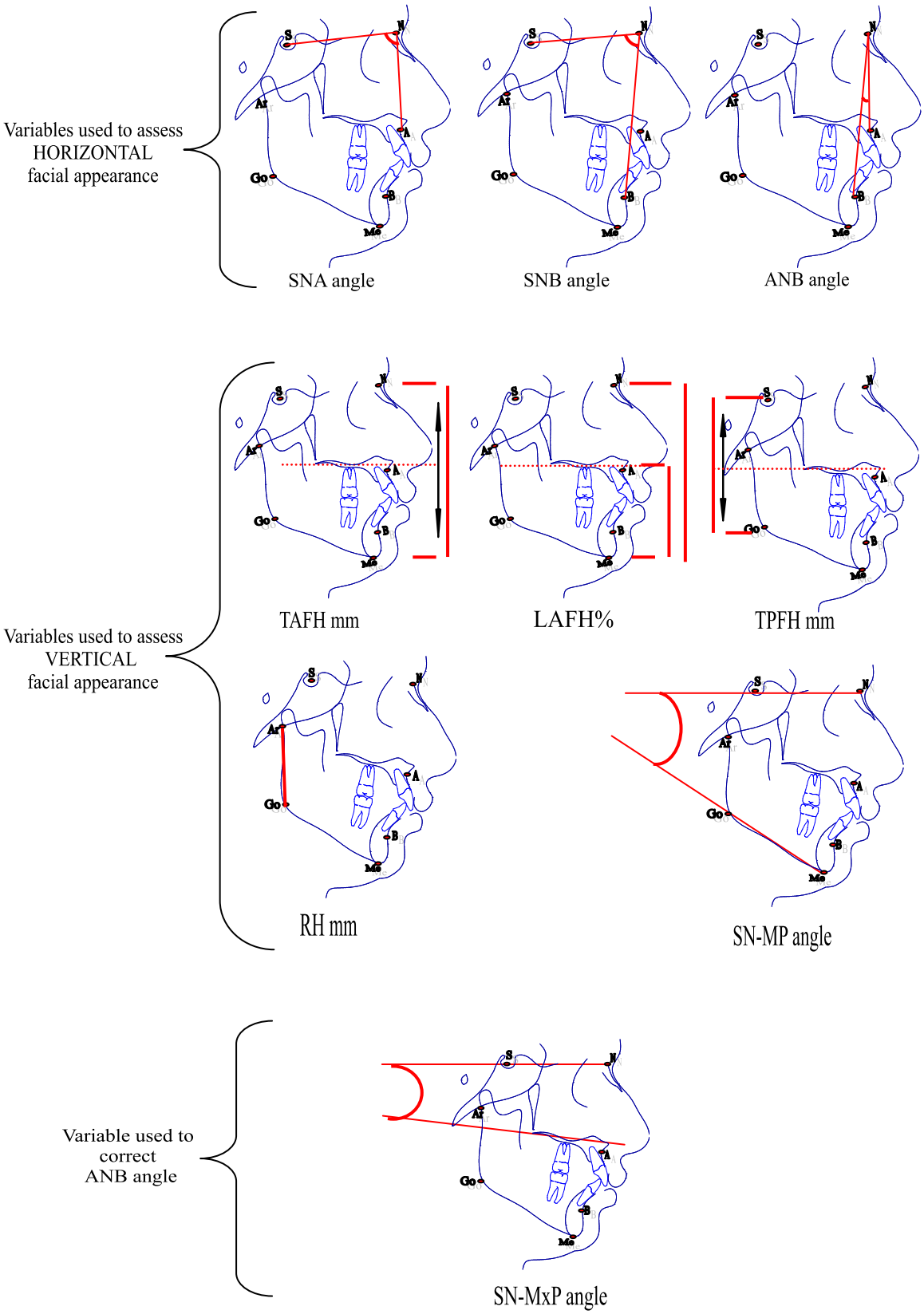


Figure 2.3: Horizontal and vertical cephalometric variables used.

2.4.4.3. Cephalometric norms

Published cephalometric norms of both the British (Bhatia and Leighton, 1993) and the Saudi (Shalhoub et al., 1987; Taibah and Feteih, 2007) Caucasian populations, matched to the mean age of the subjects, were investigated to assess the possibility of pooling the data of both groups for the current research. The mean values of both vertical and horizontal cephalometric norms of the Saudi group (Table 2.2) were within the normal range of the British norms (Table 2.3). It was therefore, considered appropriate that the patients could be pooled.

Table 2.2: Saudi cephalometric norms.

Radiographic variables	SAUDI NORMS*					
	Males			Females		
	Mean	SD	Range	Mean	SD	Range
HORIZONTAL						
SNA°	81.6	± 4.5	77.0 – 86	84.0	± 6.0	78.0 – 90.0
SNB°	78.8	± 3.7	75.0 – 82.5	81.0	± 5.8	75.2 – 86.8
ANB°	2.8	± 1.9	0.9 – 4.7	3.0	± 2.4	1.6 – 5.4
VERTICAL						
LAFH%	-	-	-	-	-	-
TAFH mm	122.8	± 8.6	114.2 – 131.4	115.8	± 7.2	108.6 – 123.0
TPFH mm	77.0	± 8.9	68.1 – 85.9	72.0	± 5.3	66.7– 77.3
RH mm	42.1	± 6.7	35.4 – 48.8	40.6	± 4.7	35.9 – 45.3
SN-MP°	33.8	± 5.0	28.8 – 38.8	28.0	± 4.7	23.3 – 32.7

SD: Standard deviation. The LAFH% was not reported in both Saudi studies *(Shalhoub et al., 1987; Taibah and Feteih, 2007).

Table 2.3: British cephalometric norms.

Radiographic variables	BRITISH NORMS*					
	Males			Females		
	Mean	SD	Range	Mean	SD	Range
HORIZONTAL						
SNA°	82.0	± 4.6	77.4 – 86.6	80.5	± 3.4	77.1 – 84
SNB°	79.7	± 4.5	75.2 – 84.2	78.0	± 3.4	74.6 – 81.4
ANB°	2.0	± 3.0	-1.0 – 5.0	2.6	± 2.4	0.2 – 5.0
VERTICAL						
LAFH%	55.0	± 1.8	53.2 – 56.8	54.6	± 1.9	52.7 – 56.5
TAFH mm	120	± 6.4	113.6 – 126.4	111	± 5.8	105.2 – 116.8
TPFH mm	80.0	± 4.0	76.0 – 84.0	71.4	± 4.3	67.1 – 75.7
RH mm	49.4	± 4.2	45.2 – 53.6	44.0	± 4.2	39.8 – 48.2
SN-MP°	31.8	± 6.8	25.0 – 38.6	34.3	± 6.0	28.3 – 40.3
SN-MxP°	6.0	± 2.4	3.6 – 8.4	8.6	± 3.4	5.2 – 12.0

*Derived from Bhatia and Leighton (1993).

2.4.4.4. Errors of the method

2.4.4.4.1. Magnification factor

The magnification factors (MF) for the EDH, WCH and RMH cephalostats were calculated using the formula: MF= Actual ruler size/Radiographic ruler size (Bergersen, 1980). For example, 10mm/ 11mm = 0.9. Both EDH and RMH cephalostats had a magnification factor of 0.9, while WCH was 0.92. All linear measurements were multiplied by their designated magnification factors to compensate for enlargement and to obtain the true cephalometric value.

2.4.4.4.2. Correction of the ANB angle

The ANB value was corrected to account for the variation in the projection of the N point between radiographs. For every degree that the SNA value was above the mean norm, 0.5 degree was subtracted from the ANB angle, and for every degree that the SNA value was below the mean norm, 0.5 degree was added to the ANB value. This correction was described by Mills (1987) and was recommended only in cases where the SN-MxP angle was within the average range.

2.4.4.4.3. Assessment of reliability and reproducibility

Reliability tests are conducted to assess random errors which are generally produced from poor quality radiographs or improper radiographic techniques that would affect landmark identification. Reproducibility measures systematic errors that depend mainly on the ability of the investigator to obtain repeated radiographic measurements (Houston, 1983). The lateral cephalometric radiographs of 25 subjects were selected at random and were traced twice on different occasions (at least two weeks apart). Both systematic and random errors were assessed using the Bland and Altman's approach (Bland and Altman, 1986) using the STATA software v10 and included:

- Lin's Concordance Correlation Coefficient: This test assesses both the correlation and the agreement between paired readings by taking into account the linearity, vertical shift and the degree of angulation of the trend line between repeated measurements. The closer this value to 1, the better the concordance between the first and the second measurements.

- A paired sample t-test: this test assesses systematic errors. If systematic biases were introduced, the paired t-test would show a significant p-value ($p \leq 0.05$) between repeated measurements (Houston, 1983).

- British Standards repeatability and reproducibility coefficient (CR): This test measures random errors by presenting an estimated value of the maximum difference between repeated measurements. The value is calculated by multiplying 1.96 by the standard deviation of the difference (SDD). The higher this value the greater the difficulty in location and measurement of a radiographic parameter.

- Limits of agreement: This parameter is presented as upper and lower values. 95% of the readings should lie between both values.

- Bland and Altman's graphs: This is a graphical representation of the systematic and random errors as well as the agreement between paired measurements. The y-axis indicates the difference between two readings plotted against the x-axis

which is the mean of the difference of paired measurements. Each graph has two mid horizontal lines and the upper and lower limits of agreement. The first mid horizontal line represents the perfect standard agreement at zero and the other mid line is the actual observed average agreement. The closer the observed line to the zero horizontal line, the less the systematic error. Furthermore, if both readings were in agreement they would lie at or near both horizontal lines. If random errors were introduced, the graph would show scattered points away from horizontal lines and beyond the 95% limits of agreement. Figure 2.4 represent examples of both systematic and random errors as viewed by the Bland and Altman graph. The results of Bland and Altman's approach are presented in Chapter 5.

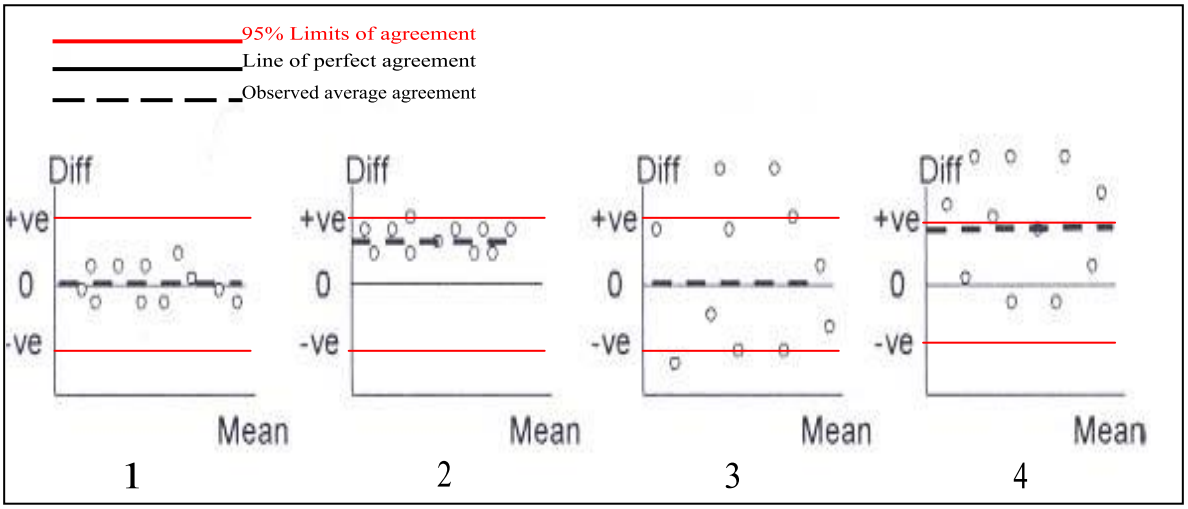


Figure 2.4: Examples of Bland and Altman graphs*. The y-axis is the difference between two readings while the x-axis is the mean of the difference of repeated measurements.* ***1)*** *No systematic errors and minimal random errors.* ***2)*** *Minimal random errors and increased systematic errors.* ***3)*** *No systematic errors and marked random errors.* ***4)*** *Both systematic and random errors are evident.*

2.5. TISSUE SAMPLES

Both microarray and qRT-PCR gene expression results of previous studies indicated a superior representation of the underlying biology when the RNA was obtained from whole fresh biopsies rather than tissue cultured cells (Zaitseva et al., 2006), frozen samples (Eikmans et al., 2000), or formalin fixed paraffin embedded (FFPE) samples (Godfrey et al., 2000). Therefore, fresh muscle biopsies were obtained from all subjects.

2.5.1. BIOPSY PROCEDURE

During the extraction of the lower third molar, the incision was extended through the inner cheek to the anterior medial portion of the superficial belly of the masseter muscle (Boyd et al., 1989). A biopsy measuring approximately 3x3x3 mm (less than 100gm) biopsy was obtained from each patient. Figure 2.5 demonstrates the location of biopsy excision.

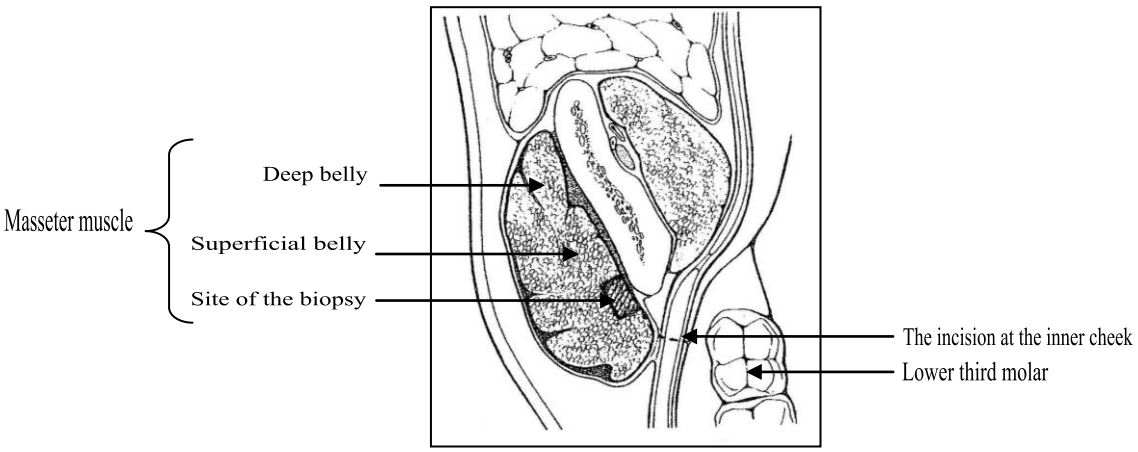


Figure 2.5: The site of masseter muscle biopsy*. A cross sectional area of the masseter muscle showing the biopsy taken from the deep, anterior surface of the superficial belly (adapted from Boyd et al., 1989).*

2.5.2. SAMPLE COLLECTION

The samples were placed immediately into a tube containing RNA stabilising reagent (RNA*later*® Tissue Storage solution supplied by Qiagen®) and kept at room temperature for no longer than 4h until transferred to a -80°C freezer. The RNA*later*® reagent inhibited the actions of any RNAses (enzymes that degrade RNA moieties).

2.6. RNA EXTRACTION

With the development of sensitive gene expression techniques, such as real-time PCR and microarrays, it has become mandatory to use high quality total RNA with no DNA contamination (Nolan et al., 2006). Several protocols were tested to generate a standardised procedure to extract good quality and quantity RNA from masseter muscle biopsies to be used for the current microarray and real-time PCR experiments (please refer to Chapter 3 where a detailed explanation of materials, methods, different protocols tested and the results of total RNA extraction procedures are presented fully).

2.7. MICROARRAY

2.7.1. DEFINITION

"A microarray is a scientific term derived from the Greek word *mikro* (small) and the French word *arayer* (arranged)" (Chaudhuri, 2005), and can be defined as microscopic elements arranged in rows and columns on a substrate surface. The microscopic elements are mainly DNA, RNA or protein probes, while the substrate could be a two-dimensional or a three-dimensional surface (Stillman and Tonkinson, 2001) made up of glass, nylon or silicon (Ramsay, 1998; Stoughton, 2005). The end product is composed of tens of thousands of probes immobilized on a small surface and is often called a chip. The whole system by which these gene chips are constructed and operated is called a platform. The current research utilises RNA gene expression. Therefore, the following sections will exclude both DNA and protein systems and will focus only on gene expression platforms.

2.7.2. GENE EXPRESSION MICROARRAY ISSUES

To accommodate the different gene expression experimental designs and to meet the requirements of microarray users, various gene expression platforms have been introduced to the market. They all share the same concept, but they have other fundamental differences. These differences were found to be in:

- The substrate surface used (Ramsay, 1998; Stoughton, 2005).
- The source (Lipshutz et al., 1999), length (Tomiuk and Hofman, 2001), and number (Alberts et al., 2007) of the RNA probes incorporated during manufacturing.
- The method by which the probe is attached to the surface substrate (Cheung et al., 1999; Lipshutz et al., 1999).
- Type of target material used (Barker et al., 2005).
- Labelling strategy (Do and Choi, 2007).

- The number of samples hybridised per chip (Hardiman, 2004).

- Normalisation, and the statistical methods used (Yauk et al., 2004; Shi et al., 2006).

With the presence of these differences, several issues have arisen indicating whether these variations would affect the accuracy, precision, specificity, and sensitivity of these systems. Accuracy has been defined as how true are the results in presenting the underlying biology (Woo et al., 2004). This issue was resolved by coupling expression profiling microarray experiments with other highly sensitive gene expression methods, such as quantitative RT-PCR (Shi et al., 2006). Precision, on the other hand, has been defined as the ability of a platform to show similar results when the experiment is repeated several times (Woo et al., 2004). This has been tested by introducing technical replicates into the design of gene expression experiments, by hybridising the same sample onto two different chips (of the same platform) followed by comparing and contrasting the results of both chips (Irizarry et al., 2005). Specificity is "the ability to identify sequences up to a certain homology" (Hardiman, 2004), and this has been found to be affected by the source, length, and the number of probes implemented during manufacturing, which may be variable between platforms (Tomiuk and Hofman, 2001; Walker et al., 2006; Alberts et al., 2007). Sensitivity was described as "the lowest concentration of the target material at which an acceptable accuracy is obtained" (Moreau et al., 2003), and this has also been found to be affected by the probe design (Stoughton, 2005).

Two other issues need to be considered when regarding microarray studies: first, the integration of microarray results from different gene expression platforms, and second, presenting and storing the high-throughput data generated by these experiments in a standardised and easy-accessible manner. The first problem has been managed by the selection of high quality microarray platforms that have been tested for their reliability and repeatability (Shi et al., 2006), as well as adapting the appropriate normalisation procedures for each platform before combining the data (Shippy et al., 2004; Carter et al., 2005). The second problem has necessitated internationally-agreed guidelines called MIAME (minimum information about a microarray experiment), which standardise the

format of publishing or exchanging microarray results (Brazma et al., 2001). As for storing microarray data, several websites have been designed to store both raw and normalised data of different gene expression platforms (Parkinson et al., 2005), while other sites can offer a re-evaluation of the original probe design of specific platforms (Liu et al., 2007). Table 2.4 summarises the microarray issues and the solutions implemented to resolve them.

Table 2.4: Microarray issues.

Microarray issues	Definition	Solutions
Accuracy	How true are the results in presenting the underlying biology?	- Confirm the results of any expression profile microarray experiment with other sensitive gene expression methods, mainly qRT-PCR
Precision	The ability of a platform to show similar results when the experiment is repeated several times	- Technical replicates
Specificity	The ability to identify sequences up to a certain homology	- Prior knowledge of the source and the whole probe sequence - Using a single long probe or multiple short probes presenting each gene
Sensitivity	The lowest concentration of the target material at which an acceptable accuracy is obtained	- Using a single long probe or multiple short probes PER gene - Amplification of the target material prior to hybridisation
Integration of microarray data	Combining the results of different studies (with similar or different microarray platforms) that are designed to answer the same scientific question	- Careful review of the probe sequence in each platform - Combine platforms with similar probe sequences - Normalise the data of each platform using its designated software prior to combining the data
Storing and publishing data	The way of storing, publishing, exchanging or presenting microarray data	- Follow MIAME guidelines - Use special websites to store the data that can be retrieved or reanalysed again

2.7.3. PRINCIPLES OF GENE EXPRESSION MICROARRAY PLATFORMS

The concept of a gene expression microarray platform has been reviewed by several authors (Cheung et al., 1999; Duggan et al., 1999; Lipshutz et al., 1999), and is summarised into five major stages:

1. Manufacturing a chip that is composed of tens of thousands of RNA probes that are immobilised onto a microscopic surface.

2. The laboratory procedure: This stage is mainly about the formation of labelled target material, hybridisation, scanning and generating raw data.

3. Gene chip quality control: This is to ensure that the experiment is conducted successfully and no technical errors are introduced.

4. Pre-processing data: This stage includes normalisation to remove any biological sources of variations and also to measure the level of expression of each gene.

5. Data analysis: This is the final stage where a series of analytical methods are conducted to make sense of the underlying biology.

Hardiman (2004) in a detailed review of the different gene expression platforms found that most of the chips that are available on the market with complete gene coverage were restricted to animals, plants, bacteria or viruses. However, some vendor corporations such as Affymetrix® were supplying gene chips with a comprehensive coverage of the human genome. Affymetrix® GeneChips® have been reported to have high accuracy (Rogojina et al., 2003), precision (Woo et al., 2004), specificity (Järvinen et al., 2004), and sensitivity (Yauk et al., 2004) when tested by several laboratories (Irizarry et al., 2005; Wang et al., 2005), scientists (Bosotti et al., 2007), and when compared to other platforms (de Reyniès et al., 2006). It was for these reasons that Affymetrix® GeneChips® were selected for this research.

2.7.4. PROPERTIES OF AFFYMETRIX® GeneChips®

2.7.4.1. The substrate

Glass surfaces are the most commonly used type of substrate as they are durable, rigid, can withstand high temperature during hybridisation, can undergo surface modification to allow for the attachment of genetic elements and have low fluorescence in order not to affect the scanned image during normalisation. Glass substrates are usually treated with special reagents to enhance hydrophobicity and facilitate covalent attachment of the genetic material to the surface. These reagents also restrict spread and interaction beteween neighbouring probes (Duggan et al, 1999). The Affymetrix® substrate is a two-dimensional 1.28 x 1.28 cm glass surface.

2.7.4.2. The probe

The term probe in this thesis describes the genetic element that is immobilised onto the substrate surface during manufacture and, according to MIAME guidelines is called the reporter. The source (Alberts et al., 2007), the length (Walker et al., 2006), and the number of probes representing each gene (Shippy et al., 2004) were all found to be different between the Affymetrix® and other microarray platforms.

2.7.4.2.1. Source of the probe

The Affymetrix® system uses the exact mRNA sequence copied from the sense strand derived from a public genetic database, such as UniGene (Schuler et al., 1996), to attach each nucleotide to the substrate and continue building an oligonucleotide probe. This procedure is called *in-situ* synthesis and produces high-density oligonucleotide platforms. This type of platform requires complex equipment to be manufactured and can only be generated by commercial laboratories such as Affymetrix® (Yauk et al., 2004).

2.7.4.2.2. Length of the probe

Probes are either short (mainly 25 bases) or long (mainly 60 bases) oligonucleotides (oligos). Affymetrix® had some technical problems with designing long probes using its own *in-situ* technique and has, therefore, decided to manufacture short probes of 25

bases. However, to improve both specificity and sensitivity, several probes are used to represent each gene and thus the set-up is a probe-set rather than a single probe.

2.7.4.2.3. Number of probes presenting each gene

Affymetrix® has designed a probe-set for each gene. The probe-set is composed of eleven probe pairs scanning different regions of the required gene. Each probe pair consists of two sequences. One sequence matches exactly the required genetic sequence and is called the perfect match (PM), while the other has an identical sequence to the PM except for a single nucleotide difference at the middle of the sequence and is called the mis-match (MM).

2.7.4.3. The method of immobilising the probe onto the substrate

Affymetrix® uses the *in-situ* synthesis technique to deposit the probes onto the substrate surface (Lipshutz et al., 1999). *In-situ* technology requires previous knowledge of the mRNA sequence of the selected gene. Once the first nucleotide is immobilised onto the glass surface, the other nucleotides are joined by covalent bonds between the 5' hydroxyl group on the backbone of one nucleotide and the phosphate group on the other nucleotide. During each round, a single nucleotide is deposited to the appropriate region of the array. To ensure this property, a protective group is added to each nucleotide on the 5' position. Once this nucleotide is chosen to be covalently attached to the other nucleotide on the surface, the protective group is converted into an active hydroxyl group in a process called deprotection. This aids attachment to the phosphate group of the previous nucleotide. The process is then repeated until the exact length of the probe is reached.

Companies use different methods of deprotection, when acid is used, the process is called chemical deprotection, while the term photodeprotection is implemented when light is used to convert the protective group into a hydroxyl group. The photodeprotection procedure can be done with or without a mask. The maskless procedure relies on using micro-mirrors to redirect light onto the appropriate parts of the glass slide. Affymetrix®, on the other hand, adapts the photodeprotection procedure using a mask and the technique is called photolithography. The mask allows the light to

pass at certain areas and not others to build up the required oligonucleotides. Each round requires a different mask, and each mask is expensive to construct.

2.7.4.4. The hybridisation system

Generally, the hybridisation system of microarray platforms could be either a one-colour or a two-colour system. This is based solely on the number of samples hybridised onto the same chip. Both systems have proven their efficiency (Irizarry et al., 2005), however, the choice of either system depends upon the experimental strategy. For example, two-colour platforms are mainly chosen when conducting time point experiments, while the one-colour chip could be used in case-control studies such as disease identification in different individuals compared to controls. The Affymetrix® platform is a one-colour system and is suited for the design of the current research.

2.7.4.5. Affymetrix® GeneChip® generations

With the discovery of new genes and improved sequencing techniques, Affymetrix® has introduced several generations of human gene expression GeneChips®. Each contains a different number of genes, similar and dissimilar probe-sets and covers different sections of the genome. To ensure maximum accuracy and precision of a microarray experiment it is recommended to use the same version of the array chip for all samples within the experiment. The most comprehensive human Affymetrix® gene expression GeneChips® were the U133 plus 2.0 array and the Exon array. Both arrays have similar accuracy (Robinson and Speed, 2007). However, the U133 plus 2.0 array showed better precision and sensitivity than the Exon array (Abdueva et al., 2007). Therefore the U133 plus 2.0 array was selected for the current microarray experiment.

2.7.5. MICROARRAY MATERIALS AND METHODS

The following sections will describe briefly the aims and objectives of each microarray step. All laboratory procedures were conducted following the Affymetrix® GeneChip® Expression Analysis Technical Manual. Summary of the kits, machines and software used are listed in Appendix B, while details of the laboratory protocol are available at Appendix C.

Affymetrix® experiments require specialised instruments, software and bioinformatics. Therefore, an Affymetrix® microarray core facility provider was used (ALMAC Diagnostics™, UK). The normalisation procedure and initial data analysis were provided by the Bloomsbury Centre for Bioinformatics (BCB)/ Department of Computer Science at UCL, while the final statistical analysis was conducted at the Eastman Dental Institute.

2.7.5.1. RNA samples used

A total of 26 RNA samples were used for the microarray gene expression technique. This represents the number of subjects recruited at the time of the microarray procedure. The RNA sample of one of the Class II patients (male) was hybridised twice onto two different GeneChips® to act as a technical replicate.

2.7.5.2. Sample preparation

- Addition of poly-A control genes into the purified RNA

The poly-A control genes are derived from *B.subtilis* and are named *dap*, *lys*, *phe*, *thr* and *trp*. They are absent in eukaryotic cells. During hybridisation, each of the five genes will bind to a special probe-set included in the original design of the Affymetrix® array chip. The reason for spiking in these genes into the RNA sample was to act as internal controls to ensure the success of the laboratory process.

- Addition of Oligo-dt primers

These primers are designed specifically with a poly-T tail. The unique design of these primers would allow them to bind to the poly-A tail of the mRNA, initiating cDNA synthesis of only the mRNA within the total RNA pool (Figure 2.6). A special promoter called T7 is attached to the oligo-dt primers. This promoter is derived from a bacteriophage and is capable of initiating *in-vitro* transcription (IVT) of only the sense strand of the cDNA to generate anti-sense cRNA which is part of the adapted strategy by Affymetrix®. This strategy was initially reported and fully described by Van Gelder and colleagues (1990).

2.7.5.3. cDNA/cRNA synthesis and amplification

Affymetrix® adopts a linear amplification strategy via IVT rather than exponential amplification. The IVT generates a single-stranded (ss) anti-sense cRNA as the final target material rather than double-stranded (ds) cDNA. However, the process of generating cRNA starts with cDNA synthesis (Figure 2.6). cDNA/cRNA synthesis can be conducted either in one-cycle or two-cycles. For samples with low starting concentration the two-cycle protocol is recommended and was used for this study.

2.7.5.4. Synthesis of biotin-labelled cRNA

The Affymetrix® system uses an indirect labelling procedure (Do and Choi, 2007). The cRNA is first labelled with a special protein called biotin during the IVT reaction at this stage, while the fluorescent dye (streptavidin-phycoerythrin –SAPE), which has strong affinity to the biotin, is incorporated later after hybridisation and during the staining step (Figure 2.6).

2.7.5.5. cRNA fragmentation

The fragmentation reaction has been optimised by Affymetrix® to generate concentrated cRNA fragments of 35 to a maximum of 200 bases. These fragments hybridise later to the probes on the array chip (Figure 2.6).

2.7.5.6. Hybridisation

Prior to hybridisation, the fragmented cRNA was mixed with a cocktail solution. The cocktail mixture included four hybridisation control genes derived from *E.Coli* (*bioB, bioC, bioD* genes) and P1 bacteriophage (cre gene). These controls are pre-synthesised as biotin-labelled cRNA transcripts in different concentrations (with the bioB having the least concentration while cre the highest). The reason for mixing these controls with each sample was to evaluate the labelling and hybridisation efficiency. The cocktail mixtures containing the fragmented cRNA and the control genes were then incubated overnight to allow the biotin-labelled fragmented targets to hybridise to their complementary probes. Any un-hybridised fragments would remain in the solution and would therefore need to be washed (Figure 2.6).

2.7.5.7. Washing and staining

- Initial washing

Initial washing was conducted to remove any non-hybridised fragments (Figure 2.6).

- Staining

The staining strategy relies on the physical properties of the biotin protein attached to the cRNA, which is capable of binding to streptavidin. A staining solution containing a set of anti-streptavidin antibodies that can bind to streptavidin was also added in order to provide an amplified signal during scanning (Figure 2.6).

- Final washing

A final wash was provided to clean the array chip from any remaining un-attached stains prior to scanning.

2.7.5.8. Scanning (data extraction)

Data extraction begins with producing an image of the array chip. The intensity of each probe on the chip can be assessed generally by visualising the different coloured features on the generated image. Black signals represent un-hybridised probes, while coloured signals represent hybridised probes. The coloured signal intensity varies from one hybridised probe to the other depending on the gene expression. The lighter the signal intensity, the greater the gene expression. The signal intensity level increases from dark-blue followed by blue, light-blue, green, yellow, orange and red, and the highest are white coloured signals (Figure 2.6). However, no conclusions can be drawn from these images. They are only used to generate special data files that are further used for data analysis.

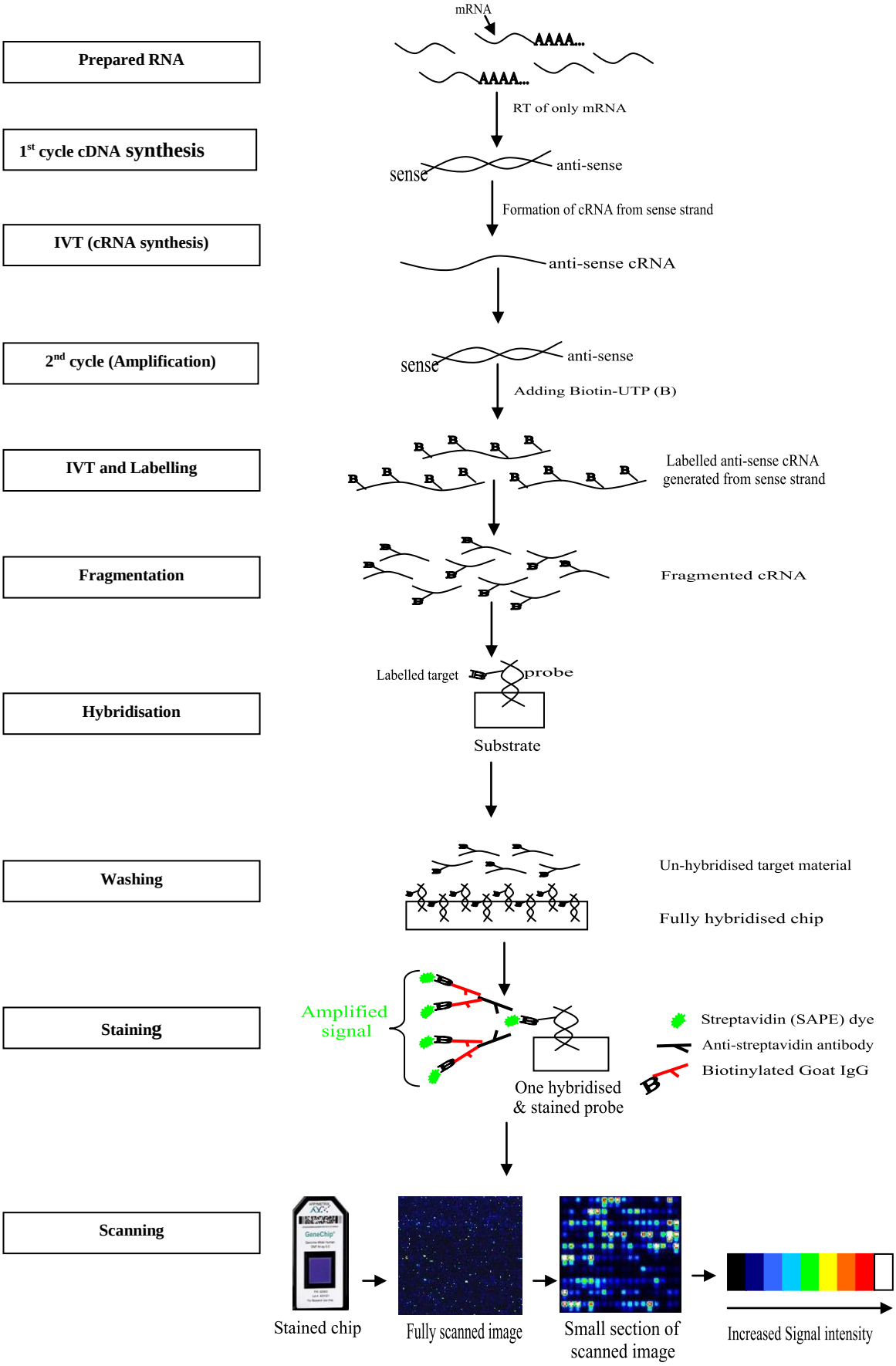


Figure 2.6: The Affymetrix® laboratory workflow.

2.7.6. GeneChip® QUALITY CONTROL

A quality control step is usually carried out prior to data pre-processing. This is to ensure an efficient labelling and hybridisation procedure as well as proper scanning (Wilson and Miller, 2005). Seven quality control measures have been used to assess the efficiency of the microarray laboratory procedure, these are explained in detail in Chapter 4.

2.7.7. PRE-PROCESSING OF DATA

Pre-processing of data was conducted at the BCB/UCL using the CEL files, Simpleaffy package (Wilson and Miller, 2005), Bioconductor v2.0 (Gentleman et al., 2004) and R v2.5.0. The process was carried out in two main steps: firstly, background adjustment and normalisation and, secondly, summarisation of probe intensity level.

2.7.7.1. Background adjustment and normalisation

The generated raw data contains non-biological sources of signals derived from the optical noise of the scanner as well as non-specific hybridisation. Both unwanted signals are called noise (Wu et al., 2004). The process of separating raw noise from the actual signal within one chip is called background adjustment while, if conducted within all chips included in the experiment, it is called normalisation. Once all systematic variations are removed between arrays, the data are viewed in different formats of plots, histograms and charts to assess and exclude any poorly correlated chips (description of the graphs, histograms and charts are presented in Chapter 4).

2.7.7.2. Summarisation of probe intensity value

The aim of this step is to generate a single expression value for each gene. Affymetrix® gene chips contain both the perfect match (PM) and mis-match (MM) probe-set design and because of this, different types of algorithms have been developed to summarise the intensity value for each gene. For example, some methods would subtract the MM values from the PM (such as the Affymetrix® default called average difference), while others would divide the values and produce a ratio. Irizarry et al., 2003 and Wu et al., 2004, have found that inclusion the MM values produces errors in the results. They have recommended summarising the PM values only and taking the log transformation which has provided better accuracy and sensitivity. The GCRMA algorithm was used

for the current experiment. This type of algorithm ignores the MM probes and relies on the average log of the PM probes, as well as using sequence information and the guanine-cytosine (G-C) base pairing to exclude any non-specific hybridisation signals. Therefore, it is called GC robust multi-array average (GCRMA) (Wu et al., 2004).

2.7.8. MICROARRAY DATA ANALYSIS

2.7.8.1. Grouping of subjects for microarray data analysis

Microarray experiments generate massive high-throughput genetic data, which makes it difficult to analyse when related to complicated craniofacial phenotypes. Therefore, a simple type of subject grouping was adopted, similar to previous genotype-phenotype masseter muscle gene expression studies, based on either vertical (Nelson-Moon et al., 1998; Suchak et al., 2009) or horizontal (Gedrange et al., 2005 and 2006; Harzer et al., 2007) facial parameters. A total of 26 patients were grouped twice based on either vertical or horizontal facial appearance. Both clinical and radiographic patterns were confirmed by two orthodontists; the researcher and an orthodontic consultant.

- Vertical grouping

Vertical facial development was assessed clinically by lower anterior face height (LAFH) in relation to mid and upper face heights regardless of the horizontal deformity and also radiographically using the TAFH (mm) (compared to British and Saudi cephalometric norms discussed earlier in Section 2.4.4.3). This classification grouped patients into 12 control (average both clinical and radiographic appearance) and 14 long face (increased both clinical lower anterior face height and radiographic TAFH) subjects. Both groups contained sufficient numbers of patients to draw conclusions in relation to vertical facial deformities.

- Horizontal grouping

Horizontal diagnosis was revealed by assessing the position of the mandible in relation to the maxilla without considering vertical facial appearance, both clinically and radiographically (using the ANB angle). This generated three groups including 11 controls (the position of the mandible was at or slightly behind the upper jaw and a radiographic ANB angle 1-4°), 5 Class II (clinical retrognathic appearance and a

radiographic ANB angle of > 4°) and 10 Class III (clinical prognathic appearance and a radiographic ANB angle of < 0°) individuals. Both the Class III and control groups contained sufficient sample size to draw conclusions from the microarray analysis in relation to prognathic appearance. However, the Class II pattern had a reduced number of patients (5) compared to the initial (11 patients) and retrospective (7 patients) calculated sample size. Therefore, caution was recommended when assessing interpretation of data with regard to retrognathic deformity.

2.7.8.2. Generating differentially expressed gene lists

Differentially expressed genes are often determined by using multiple hypothesis testing and a cut-off p-value of 0.05. This would usually generate large numbers of false significant results. A Benjamini-Hochberg test or a Bonferroni correction is often used to correct for multiple testing. However, the criteria are very stringent for microarray data (Leung and Cavalieri, 2003). Microarray data analysis for the current research was conducted using the GCRMA files and LIMMA package (Bioconductor v2.0) (Gentleman et al., 2004). This type of analysis provides a type of t-test modified to accommodate the large amount of data. The p-values were calculated without any correction and the false significant results were minimised by choosing a cut-off p-value of 0.001. Any gene with an uncorrected p-value ≤ 0.001 was considered differentially expressed between the deformity and the control group. The analysis was conducted twice: first, to generate a differentially expressed gene list of the vertically classified groups (Long face vs. control); second, to generate a differentially expressed gene lists between horizontally classified groups (control vs. Class II vs. Class III).

2.7.8.3. Filtering the data

A forward stepwise logistic regression analysis (SPSS v14) was used to narrow down both generated gene lists from the microarray experiment. Genes with a p-value of $\leq$ 0.001 were considered significantly different. The results of the microarray data analysis are presented in Chapter 4.

2.8. QUANTITATIVE RT-PCR

2.8.1. INTRODUCTION

In general, PCR techniques have been designed to amplify only a DNA sequence. Therefore, if RNA is the target material it has to be converted into complementary DNA (cDNA) in a process called reverse transcription (RT) prior to any application. In gene expression experiments the generated cDNA is further amplified via the PCR technique and the whole process is called reverse transcription polymerase chain reaction (RT-PCR) (Robert and Farrell, 2005).

2.8.2. THE CONCEPT OF REVERSE TRANSCRIPTION (RT) AND THE POLYMERASE CHAIN REACTION (PCR)

The RT step is a single reaction conducted mainly at room temperature using a special enzyme called reverse transcriptase, usually random primers are used. These primers are designed to flank different regions of the genetic sequence and initiate the conversion of most of the RNA into cDNA (Nolan et al., 2006). The PCR step is exponential and is conducted in several reactions to generate millions of copies of the required sequence using a set of gene specific primers (GSP), an enzyme, a buffer, dNTPs and a thermocycler machine.

The primers are synthetic short single strand oligos (mainly 25-30 bases) targeting only the required gene to be amplified. Two primers are often designed to determine the beginning and the end of the region of interest. The enzyme on the other hand, is called DNA polymerase and is capable of duplicating the region of interest. The Taq polymerase is an example of a naturally derived enzyme from a bacterium called *Thermus aquaticus* and is often used. The buffer provides a suitable chemical environment for the enzyme to work, while the dNTPs (deoxyribonucloside triphosphate) are the building blocks used by the polymerase enzyme to build up the required sequence during the reaction. The PCR reaction is conducted in cycles (mainly 35-40 cycles), with each amplification cycle undergoing three phases, namely denaturation, annealing and elongation. Each phase requires a different temperature as provided by a thermocycler machine. The temperature varies from one protocol to

another depending on the enzymes and primers used. Figure 2.7 describes the basic steps for RNA amplification using the RT-PCR technique.

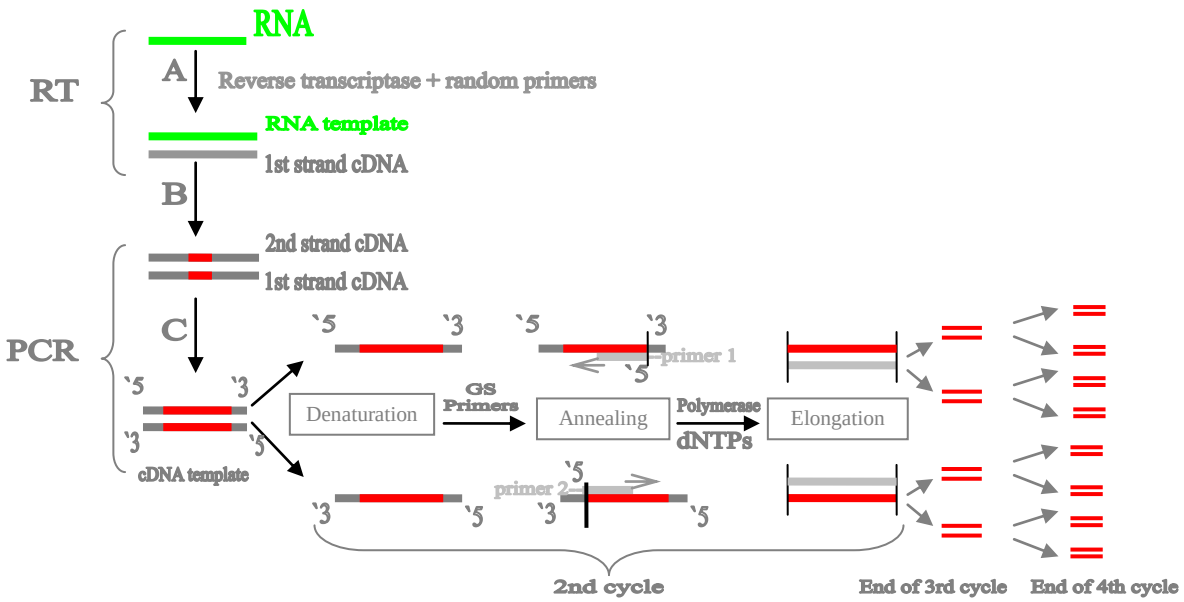


Figure 2.7: General principles of the reverse transcription (RT) and the PCR technique. A) *The RT step uses the reverse transcriptase enzyme and random primers to generate a hybrid of the RNA template and the 1st strand cDNA.* ***B)*** *During the 1st PCR cycle the RNA template is degraded and a hybrid of both 1st and 2nd strands-cDNA are formed.* ***C)*** *The 2nd PCR cycle starts by using the ds-cDNA as a template, gene specific (GS) primers and the DNA polymerase enzyme to amplify the gene of interest (marked in red). The PCR step has 3 phases that occur in each cycle, for demonstration purposes, the 3 phases are only described at the 2nd cycle. By the end of the 2nd cycle the required gene will amplify by 2 copies, 4 copies in the 3rd cycle, 8 copies in the 4th cycle, and so on until a certain number of cycle n.*

2.8.3. QUANTITATIVE vs. END POINT TO MEASURE GENE EXPRESSION

The level of expression of a particular gene is detected by incorporating a special fluorescent dye during the cDNA amplification. The higher the gene expression, the greater the fluorescence intensity. The gene expression intensity can be detected either at the end of the PCR procedure or during amplification. If the standard PCR technique is used for amplification, the level of expression is determined at the end of the reaction by running the PCR products on an agarose gel, a procedure called end-point measurement while, if the level of expression is measured during the amplification phase of the PCR, the procedure is said to be in real-time. There is a direct relationship between the level of expression of a particular gene and the amplification cycle at which the fluorescence will occur (i.e. the earlier the cycle at which the fluorescence will occur

the higher the gene expression). This relationship cannot be detected by the end-point procedure. Therefore, real-time PCR has the advantage of being more sensitive in detecting minute variations in the level of expression (Schmittgen et al., 2000). This is achieved by using a special thermocycler that is equipped with a sensitive camera and a monitor to display the accumulated fluorescence of the gene in each amplification cycle.

Real-time PCR provides a quantitative measurement of the expression of a particular gene and it has, therefore, been called the quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). The qRT-PCR has been reported with high agreement and concordance with the Affymetrix® gene expression chips (Shi et al., 2006; de Reynie's et al., 2006) and was, selected therefore, as a sensitive gene expression technique parallel to microarrays to assess genes of interest.

2.8.4. TYPES OF qRT-PCR

Two types of qRT-PCR are available, absolute and relative quantification. Both types have been reported with accurate, precise and sensitive results (Cikos et al., 2007). However, the selection of one type over the other is governed by the design of the experiment. Absolute quantification provides a precise measurement by comparing the level of expression of an unknown sample to another standard reference template with known gene expression, such as the detection of viral load in a sample. The procedure is called the standard curve method. Relative quantification, on the other hand, is used to answer most clinical questions and provides a relative quantification of the gene expression of one sample compared to a calibrator or a control sample, as in time-point experiments or case-control studies. This procedure relies on the differences in the crossing threshold (Ct) cycle value between a sample and a control. The Ct value is the cycle at which a significant increase in the fluorescence of a particular gene is detected. Therefore, the procedure is called the comparative Ct method (Cikos et al., 2007). The relative qRT-PCR (comparative Ct method) was adopted for this research.

2.8.5. qRT-PCR PROTOCOL

The following sections will describe the materials and methods used for the qRT-PCR experiment. Summary of the kits, machines and software used are listed in Appendix B while details of the laboratory protocol are available at Appendix C.

2.8.5.1. RNA samples used

The 26 RNA samples used for the microarray experiment as well as three extra RNA samples purified from newly collected samples (total 29 samples) were used for the qRT-PCR procedure.

2.8.5.2. cDNA synthesis

cDNA synthesis and amplification can be conducted either in one-step or two-steps. In the one-step method, all the reagents and primers of both the reverse transcription and the relative quantification real-time PCR amplification steps are mixed together in a single tube. Having all the reagents mixed in the same tube produce less contamination and saves time. However, the reaction is not optimised and the single enzyme (which performs both reverse transcription and amplification) is less efficient than having two separate enzymes performing under different optimal conditions (Easton et al., 1994). In the two-step procedure, one enzyme is used to perform the reverse transcription to generate cDNA, and the second enzyme is used to perform the amplification. Each step is performed under different optimal conditions. Although the two-step method is more time consuming than the one-step procedure, it is more flexible and sensitive when used for research purposes (Nolan et al., 2006). Therefore, the two-step method was selected for the relative quantification real-time PCR technique.

2.8.5.3. Endogenous reference gene

The use of endogenous reference controls such as housekeeping genes in qRT-PCR reactions is a routine procedure. The expression of these genes often remains stable in all cells under different biological and experimental conditions (Robert and Farrell, 2005). Based on these grounds, endogenous genes are used to demonstrate that the overall expression of a target gene is not a result of variations in the RNA content, degradation or experimental errors and is a true biological variation. The most commonly used housekeeping genes are the ß-actin, ß2-microglobulin (ß2M) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The expression of some of these genes has been found to be variable in different tissues (Lee et al., 2002) and it is important to select the appropriate control gene for the tissue of interest. GAPDH has been found to be the least variable among different tissues (Lee et al., 2002) and more stable in skeletal muscles of patients with different age categories (Touchberry et al.,

2006) and under different environmental conditions (Jemiolo and Trappe, 2004). Therefore, the GAPDH gene was selected for the current qRT-PCR experiment.

2.8.5.4. Fluorescent dye chemistry

Fluorescent dyes are classified based on their binding specificity into either specific or non-specific dyes (Busten and Nolan, 2004). The non-specific dyes, for example the SYBR® Green dye, are added as a separate reagent into the PCR mixture and bind to any double stranded genetic molecule that is generated during the amplification reaction. This type of dye has the advantage of being cheap and can be incorporated into any optimised PCR reaction. However, it can bind to any double strand target including primer-dimer (two primers attached to each other) which might affect its specificity. The specific dyes, on the other hand, are included within the original design of the primers. The design would include two gene specific primers plus a fluorescent probe and the whole design is called an assay. Different types of assays are available commercially, and their differences were observed in the shape of the fluorescent probe. Some assays have linear probes such as the TaqMan®, while others contain looped probes such as Beacons® and Scorpions®. The unique chemistry used to generate the specific dyes is more expensive than that for the non-specific ones (Busten and Nolan, 2004) but the earlier provide higher specificity, sensitivity and accuracy (Levesque-Sergerie et al., 2007). Furthermore, the TaqMan® assay is the most commonly used type of the specific dye and has shown high agreement with the Affymetrix® platform (Shi et al., 2006). Therefore, the TaqMan® chemistry was chosen for the current research (Figure 2.8).

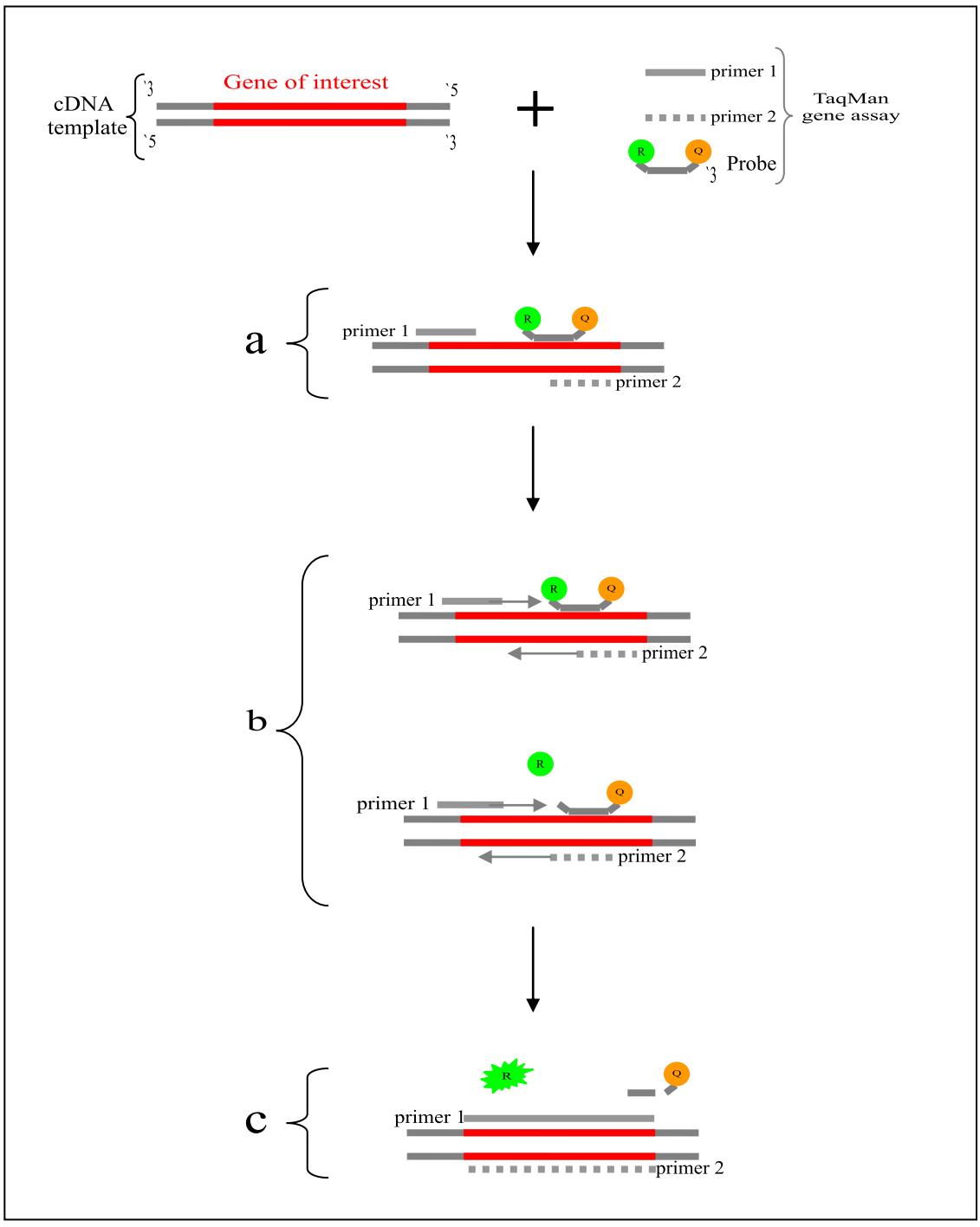


Figure 2.8: The TaqMan® chemistry. *The linear probe included in the TaqMan® assay is an oligonucleotide sequence that is designed to anneal close to one of the primers on one of the cDNA strands. One end of the probe (usually the 5') contains a fluorescent reporter dye (R), while the other end (3') has a quencher (Q). As long as the reporter remains on the same oligonucleotide sequence and in close proximity to the quencher, no fluorescence will occur.* ***a)*** *Both primers as well as the probe are designed to attach to the designated gene and are in close proximity to each other.* ***b)*** *Once the polymerase enzyme extends the primer, it will encounter the probe on the 5' end and will hit and release the reporter dye (R) into the solution.* ***c)*** *Following the release of the reporter dye, it is no longer quenched and fluorescence will occur. This fluorescence will increase by each PCR amplification cycle.*

2.8.5.5. Normalisation and calculation of gene expression values

The normalisation process is required to remove any source of variation in RNA concentration and cDNA preparations between different samples. This procedure, and the calculation of the final gene expression value, is not a direct process and require the use of the Ct value in several equations. These equations have been described in detail by Livak and Schmittgen (2001) and will be discussed briefly in the following sections. All Ct values were exported from the qRT-PCR machine, and were transferred to an Excel spreadsheet where the gene expression value was calculated using the final equation $2^{-\Delta\Delta Ct}$. Figure 2.9 shows an example of a qRT-PCR amplification plot by which the Ct values were derived.

- Step 1 (repeatability)

  Objective: To ensure high precision, both the target and the reference genes were experimented in quadruplicates. This produced several Ct values which were summated to produce an average Ct value obtained for both genes.

  Equation: = Average Ct of reference gene

  Average Ct of target gene

- Step 2 (normalisation)

  Objective: To normalise the average Ct of the target gene in relation to the average Ct of the reference gene of the same sample thereby producing the ΔCt

  Equation: = Average Ct target – Average Ct reference = ΔCt

- Step 3 (calibration)

  Objective: One of the control samples, which had average vertical and horizontal craniofacial features, both clinically and radiographically, was selected as the calibrator sample (C). This was to produce ΔΔCt for each sample. During calibration, the calibrator sample was also calibrated to itself to produce a value of zero. The reason for generating a zero value for the calibrator sample was to produce a value of one when used in the next equation, and one would act later as a baseline for relative gene expression comparison.

  Equation: = ΔCt target – ΔCt calibrator = ΔΔCt

- Step 4 (gene expression value)

  Objective: The fourth equation was derived from the belief that each gene was doubled during each amplification cycle, assuming 92-100% amplification efficiency (Livak and Schmittgen, 2001). The equation transferred the calibrator value into 1 which acted as a baseline for gene expression comparison. The generated gene expression values of the other samples which were higher or lower from the baseline were considered a fold change. The results were then used for further data analysis to assess the significance of the difference.

  Equation: = $2^{-\Delta\Delta Ct}$

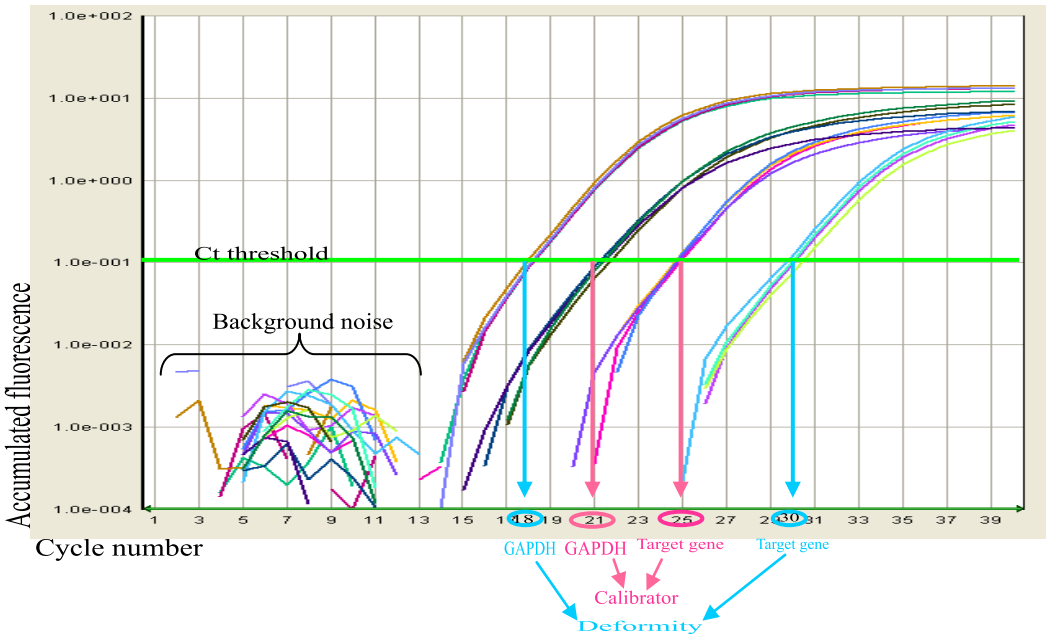


Figure 2.9: An example of calculating the relative gene intensity value using the $2^{-\Delta\Delta Ct}$ equation. *Both GAPDH and target gene for each sample have been tested in quadruplicate reactions. These are presented as 4 lines in close proximity. The background noise is the level where no fluorescence is seen, usually up to cycle 13. The Ct threshold is the cycle at which a detectable increase in the fluorescence is observed. The later the Ct value the lower the gene expression, as in the deformity target gene, occurring at almost Ct 30. The Ct values obtained from the quadruplicate reactions are summated to produce the average Ct for each gene. For example, to calculate the gene intensity value: 1) Calculate the ΔCt (deformity=30-18=12; calibrator=25-21=4); 2) Calculate ΔΔCt (deformity=12-4=8; calibrator= 4-4=0); 3) Calculate $2^{-\Delta\Delta Ct}$ (deformity=2^{-9} =0.004; calibrator=2^{0} =1). Interpretation of the data indicates that the deformity patient has a relatively lower gene expression (0.004 fold change) compared to the calibrator sample.*

2.8.6. qRT-PCR DATA ANALYSIS

All qRT-PCR data analysis will be discussed in Chapter 6, including the various patient groupings and the different types of statistical analyses employed.

2.9. SUMMARY OF THE DESIGN OF THE STUDY

A summary of the design of the current research is presented in Table 2.5.

Table 2.5: Design of the current study.

Materials	Design
ETHICS	
Ethical approval	Three sites (EDH, WCH, RMH)
SUBJECTS	
Type of study	Case control study (Deformity vs. Control)
Types of craniofacial deformities included	Class II, Class III and long faces
Experimental variable	Gene expression level
Diagnostic tools	Lateral cephalometric radiographs
TISSUE SAMPLES	
Tissue of interest	Masseter muscle
Type of tissue used	Fresh muscle biopsy
Sample storage	RNA*later*®
MICROARRAY	
Platform	Affymetrix®
GeneChip® generation	HG U133 plus 2.0 array
qRT-PCR	
Type of RT-PCR	Relative quantification
cDNA synthesis	Two-step procedure
Fluorescent dye chemistry	TaqMan® assay
Endogenous reference gene	GAPDH
Comparative Ct method	$2^{-\Delta\Delta Ct}$

Chapter 3. Optimisation of total RNA extraction protocol from fresh human masseter muscle biopsies

3.1. INTRODUCTION

Several protocols for RNA extraction from human masseter muscle tissue have been reported in the literature (Monemi et al., 1996; Price et al., 1998; Singh et al., 2000; Gedrange et al., 2005). Most of these protocols are based on the Guanidinium-Acid-Phenol-Chloroform method originally described by Chomczynski and Sacchi (1987). Although this method has provided good RNA yield (Deng et al., 2005), high DNA contamination has also been reported when compared with other RNA extraction methodologies (Deng et al., 2005; Schagat et al., 2008). Both gene expression microarray (Hatfield et al., 2003) and quantitative RT-PCR (Peters et al., 2004) require high quality RNA with no DNA contamination. Therefore, it was important for the current study to assess a range of RNA purification techniques, procedures and reagents to optimise the RNA extraction protocol, with minimal genomic DNA contamination.

3.2. STEPS TO ELIMINATE DNA CONTAMINATION

Various procedures have been recommended to eliminate DNA contamination from RNA samples. These were vigorous disruption and homogenisation (Berglund et al., 2007), the use of silica membrane spin columns (Deng et al., 2005) and application of DNase digestion reagents (Peters et al., 2004).

3.2.1. DISRUPTION AND HOMOGENISATION

Disruption is performed to break down the cell membrane, nuclear membrane and connective tissue proteins to release the genetic material (DNA and RNA) within the cell, and is often conducted using lysing buffers (chemical disruption). Homogenisation is the process of reducing the viscosity of the lysate solution which aids the release of the DNA and RNA from the surrounding protein membranes. Homogenisation is mainly performed using different types of grinding machines (mechanical homogenisation) (Robert and Farrell, 2005). Depending upon the nature of the tissue, some would require disruption and light homogenisation, while other tissues with high connective tissue content would necessitate disruption combined with vigorous crushing. Skeletal muscles have significant connective tissue (extracellular matrix) and protein content (contractile proteins of the muscle fibres) and it was therefore recommended to use both chemical (lysing buffers) and aggressive mechanical

(crushing and grinding) techniques to release the RNA material (Robert and Farrell, 2005).

The Guanidinium-Acid-Phenol-Chloroform method (Chomczynski and Sacchi, 1987) that was adopted by previous masseter muscle researchers was based on different types of chemical lysing buffers combined with either mortar and pestle or standard homogenisers to perform mechanical disruption and homogenisation (Price et al., 1998; Singh et al., 2000; Gedrange et al., 2005 and 2006; Harzer et al., 2007). However, the use of standard homogenisers was less efficient when compared to a vigorous disruption procedure including vessels containing ceramic beads placed in a special reciprocating machine (Berglund et al., 2007). The vigorous protocol conducted by Berglund and colleagues (2007) was performed on fibrotic skin tissue using ceramic beads (lysing matrix D®) placed in a special device called the FastPrep® machine, with a speed of six metres per second for 40 seconds and placed on ice for 5 minutes, repeated three times. The FastPrep® machine is a reciprocating device which can accommodate twelve lysing matrix vessels, and has a rotation range of 4-6.5 metres per second (Figure 3.1). Furthermore, several types of lysing ceramic and stainless steel beads are available on the market and it was not known whether muscular tissue would behave in the same manner as skin when the lysing matrix beads and the FastPrep® machine were used for disruption and homogenisation. Therefore, it was decided to test different types of beads and various speed settings of the FastPrep® machine to optimise the disruption and homogenisation of masseter muscle tissue samples.

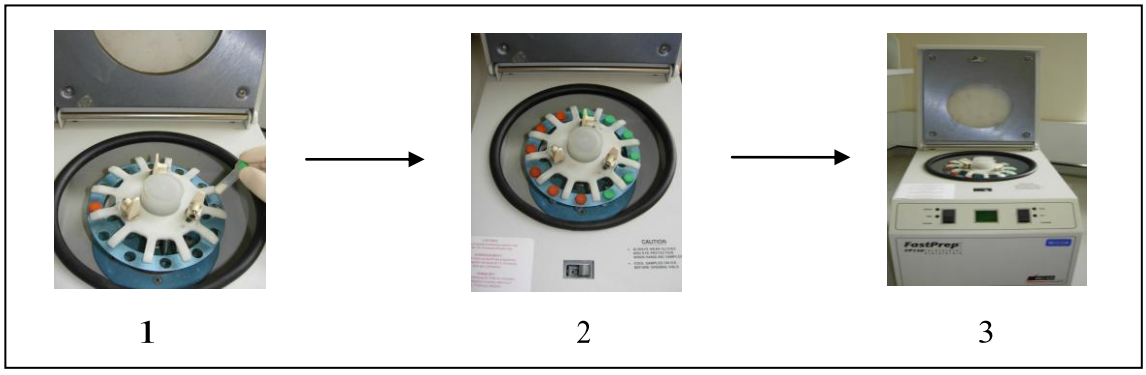


Figure 3.1: The FastPrep® machine. 1) *Applying different lysing matrix vessels, which contain the lysing buffer, the beads and the sample, into the reciprocating device.* ***2)*** *All twelve slots of the FastPrep machine are filled with the lysing matrix vessels.* ***3)*** *Ready for disruption and homogenisation.*

3.2.2. THE PURIFICATION TECHNIQUE

The aim of the purification technique is to separate RNA from DNA and protein following the disruption and homogenisation procedure (Robert and Farrell, 2005). The Chomczynski and Sacchi (1987) method relies on the organic solvent properties of acid-phenol and chloroform to separate the lysate solution based on density, leaving two main layers within the same tube; the aqueous phase which contains RNA and the organic phase which contains DNA and protein. An alternative and more recently developed silica membrane technology has been reported to produce less DNA contamination (Deng et al., 2005; Schagat et al., 2008). This technology incorporates a silica-gel-membrane spin column® which has selective binding abilities that can attract and bind RNA to the membrane. The filtration of DNA and proteins through the membrane can then be discarded. Furthermore, the working time of the Guanidinium-Acid-Phenol-Chloroform procedure was around six hours compared to one hour of the silica membrane technology (Santiago-Vázquez et al., 2006). Therefore, the silica membrane technology was selected to purify high quality RNA samples with minimal DNA contamination.

3.2.3. DNase DIGESTION

Although silica membrane technology markedly reduces the DNA contamination, DNase digestion reagents were still provided as an optional step that can be used separately with any extraction procedure. DNase digestion can be performed on-column during RNA purification or as a separate step following RNA elusion (Bustin, 2002). Samples without DNase digestion were reported with 25% DNA contamination, while the use of both procedures (on-column and after elusion protocol) markedly eliminated DNA to leave less than 2% contamination (Peters et al., 2004). However, for samples with expected low RNA yield it is preferable to use the on-column rather than the post-elution digestion procedure (Bustin, 2002). Therefore the on-column DNase digestion method was chosen in the present study.

During the course of this study, a recently published human masseter muscle study (Suchak et al., 2009) has successfully used ceramic beads (lysing matrix D®), the FastPrep® machine and silica membrane technology to purify total RNA from fresh human masseter muscle biopsies which was then used for quantitative RT-PCR gene

expression. However, the machine settings were not reported and no DNase digestion step was performed.

The aims of the above newly developed protocol within this research were therefore, to use all possible means of DNA elimination from fresh human masseter muscle biopsies, as both microarray and quantitative RT-PCR gene expressions require high quality and purity RNA samples. This was achieved by:

1. The use of different types of beads including the lysing matrix beads® to ascertain the best type that can perform proper disruption and homogenisation of masseter muscle biopsies.

2. The use of different settings of the FastPrep® machine to provide sufficient disruption for muscular tissue without RNA degradation, that may occur as a result of heat generated from the crushing process.

3. Assess the use of the silica membrane technology (RNeasy® mini kit from Qiagen™) with and without DNase digestion step.

3.3. MATERIALS AND METHODS

The following sections will describe briefly the materials and methods used for total RNA extraction. All laboratory procedures were conducted following the RNeasy® mini kit technical manual. However, optimisation procedures and modifications within the original RNeasy® mini kit protocol will be discussed in details. Summaries of kits and machines used are listed in Appendix B, while details of the final RNA extraction protocol are available at Appendix C.

3.3.1. INHIBITION OF RNase ACTIVITY

RNases are a group of enzymes that are present in almost all cells and can degrade RNA. The RNase activity should be inhibited at all stages of RNA preparation (Robert and Farrell, 2005). All working surfaces and instruments (e.g. centrifuge, pipettes and bottles) were cleaned with special wipes (RNase*Zap*® wipes) which remove RNases. Furthermore, the tips, centrifuge tubes and water used were also RNase free.

3.3.2. SOURCES OF THE SAMPLES USED

A total of six masseter muscle biopsies collected from four subjects were used to standardise the total RNA extraction protocol. Two patients had both right and left biopsies, while the other two patients had the biopsy taken from one side only.

3.3.3. THE AMOUNT OF STARTING MATERIAL

Based on the binding capacity of the spin columns of the RNeasy® mini kit, the required amount of starting tissue was not more than 30 mg. Using microscissors and stainless steel tweezers, each muscle biopsy was cut, weighed (30 mg each), and then transferred into a clean tube without any RNA*later*® to be disrupted and homogenised.

3.3.4. DISRUPTION AND HOMOGENISATION

3.3.4.1. Chemical disruption

Following the manufacturer's protocol, chemical disruption was performed using the RLT® lysing buffer mixed with 2-Mercaptoethanol. Both materials are strong reagents that can dissolve proteins to release the RNA from the cells. Furthermore, the RLT® buffer included guanidinium thiocyanate which inhibits RNase activity.

3.3.4.2. Mechanical homogenisation

- Optimisation procedure

Three different types of beads (lysing matrix D®, lysing matrix A® and cold stainless steel beads) (Figure 3.2), and three different settings of the FastPrep® machine (1 round 40s, 1 round 20s and 2 rounds of 20s) were tested to standardise the disruption and homogenisation procedure (Table 3.1). The stainless steel beads (supplied by Qiagen®) were kept cold in a fridge prior to use, in order to reduce the heat generated in the reciprocating machine.

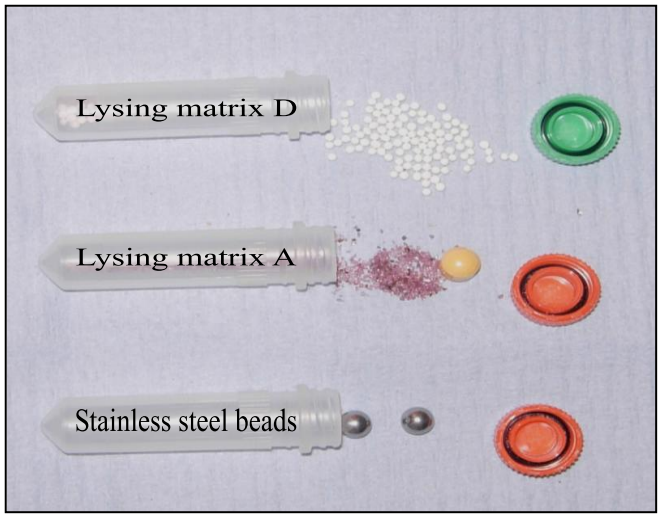


Figure 3.2: The various types of beads used for optimising the disruption and homogenisation procedure. *The lysing matrix D contains small 1.4mm white ceramic spheres. The lysing matrix A contains one ¼'' ceramic bead with grain-like garnet matrix. The third tube contained two stainless steel beads.*

Table 3.1: Nine different protocols tested to standardise the disruption and homogenisation method.

Beads type	Machine settings				Cooling down
	Protocol no	No of rounds	Speed/ round	Time	
Lysing matrix D (1.4mm ceramic spheres)	1	1 round	6	40s	Once, 5m after disruption
	2	1 round	6	20s	Once, 5m after disruption
	3	2 rounds	6	20s	Twice, 5m between and after rounds
Lysing matrix A (one ¼'' ceramic bead with garnet matrix)	4	1 round	6	40s	Once, 5m after disruption
	5	1 round	6	20s	Once, 5m after disruption
	6	2 rounds	6	20s	Twice, 5m between and after rounds
Stainless steel (StSt) (2 stst beads)	7	1 round	6	40s	Once, 5m after disruption
	8	1 round	6	20s	Once, 5m after disruption
	9	2 rounds	6	20s	Twice, 5m between and after rounds

s: Second. m: Minute.

3.3.5. RNA PURIFICATION

The lysate solution generated from the disruption and homogenisation was spun down and then transferred to a clean tube. To initiate the selective binding of RNA to the silica membrane, the lysate solution was mixed with an equal amount of 70% ethanol.

3.3.5.1. Binding

The lysate was passed through the column membrane by centrifugation. The RNA was bound to the filter membrane, while the flow-through supernatant containing the DNA and protein contaminants passed through the filter and collected in a tube which was then discarded.

3.3.5.2. Elimination of DNA contamination

- Optimisation procedure

To test whether a DNase digestion step was required for the RNA purified from masseter muscle tissue, each of the first three protocols described earlier in Table 3.1 was repeated twice using the same tissue samples; once with DNase digestion and secondly without DNase digestion. The DNase digestion step was performed using the manufacturer's protocol of the RNase-free DNase set® kit. The DNase digestion reagent was kept on-column for 15 minutes at room temperature.

3.3.5.3. Washing

Following the manufacturer's protocols for both the with and without DNase digestion, the filter membrane was washed and centrifuged several times using different buffers to remove any remaining DNA or protein contaminants attached to the membrane. The RNA remained attached to the silica membrane.

3.3.5.4. Elusion

30µl of RNase free water was applied to the membrane for five minutes at room temperature to dissolve the bound RNA. This was followed by centrifugation at maximum speed to elute the total RNA in a clean tube. The total RNA was distributed into 6 equal aliquots, each containing 5µl and was kept at -80°C for further use.

3.3.6. RNA QUALITY CONTROL

3.3.6.1. RNA quantity

The quantity of RNA was assessed using a spectrophotometer which measures the optical density (OD) of the RNA at a wavelength of 260nm. The RNA concentration (µg/ml) was then calculated using the OD260 value multiplied by the dilution factor (dilution factor = total volume/ RNA volume).

3.3.6.2. RNA purity

The purity of RNA was routinely assessed by the A260/A280 ratio that is derived from the spectrophotometer. The A260/A280 ratio is generated from the light absorbance of the genetic material at a wavelength of 260nm and the protein at 280nm. Acceptable RNA purity was represented by a ratio of 1.68-2.06. Samples having an A260/A280 ratio lower than 1.68 (protein contamination) or higher than 2.06 (DNA contamination) were excluded from the experiments.

3.3.6.3. RNA quality

The quality of RNA was assessed using an Agilent 2100 Bioanalyser® and the RNA 6000 Nano LabChip kit (detailed protocol at Appendix C). The Nano chip contains microcapillary channels that are filled with gel. Once the RNA has been loaded into the chip, the nucleic acid is separated based on size by a voltage-induced system and fluorescence occurs by incorporating a fluorescent dye during the preparation (Schroeder et al., 2006). The fluorescence is laser-induced by a Bioanalyser machine. The quality of total RNA was assessed by observing the 18 and 28 subunits (S) of the ribosomal RNA (rRNA) that was viewed as an image on the computer using special software supplied by the same company. The image was composed of two components, the y-axis which is the fluorescence (Fu) and the x-axis which is the size of the fragment (nucleotide –nt) and was presented as a line with several peaks. Figure 3.3 describes the basic components of an electropherogram.

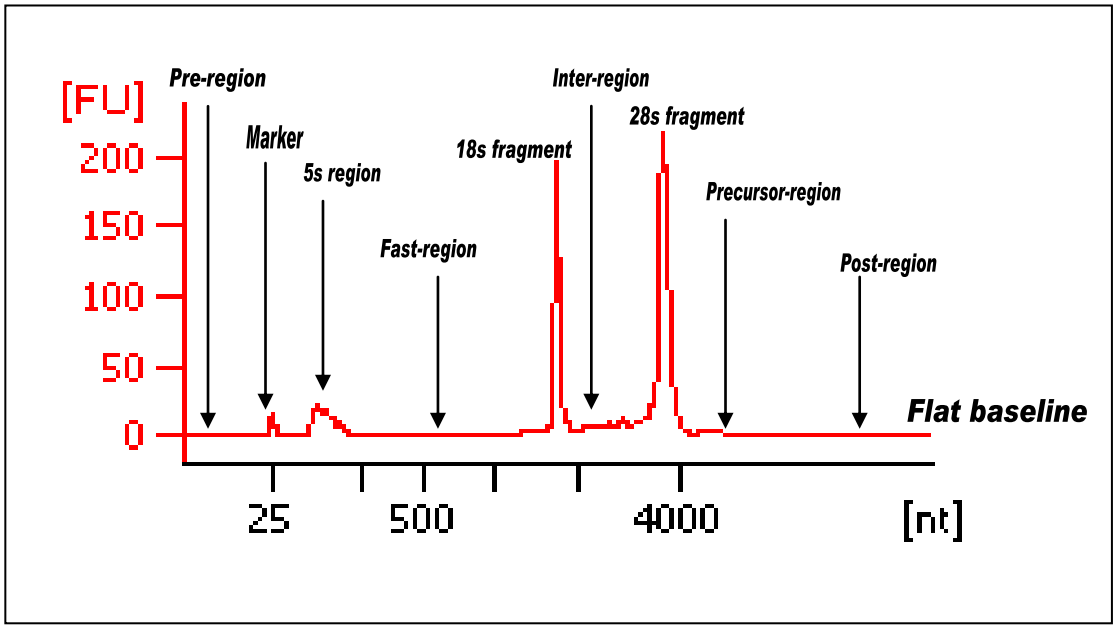


Figure 3.3: Components of good RNA quality as viewed by the electropherogram image. *Fu, fluorescence; nt, nucleotide.*

The amount, quality and purity of the total RNA sample has a large impact on the shape, length and the position of the peaks including the 18S and 28S of the rRNA peaks generated on the image. For example, the higher the amount and yield of the total RNA, the greater the 18S and 28S rRNA peaks (low amounts of total RNA are presented as short peaks). Following the manufacturer's guidelines for electropherogram image interpretation, the DNA contamination could be seen as a peak signal at the inter-region or the precursor-region. RNA degradation often occur as several well-defined peaks at the fast, inter or precursor regions. Elevation of the baseline, particularly at the inter-region would indicate partially digested RNA. Any peaks at the pre- or post-region or rising of the marker signal were not considered critical. Any samples with obvious DNA contamination or RNA degradation were excluded from further experimentation. Figure 3.4 illustrates some examples of the good and poor RNA quality outputs ranging from a scale of 1-10, where 10 was the best quality. Samples with electropherogram similar to the scale 1, 2, 3, 4 and 5 were excluded from the experiments.

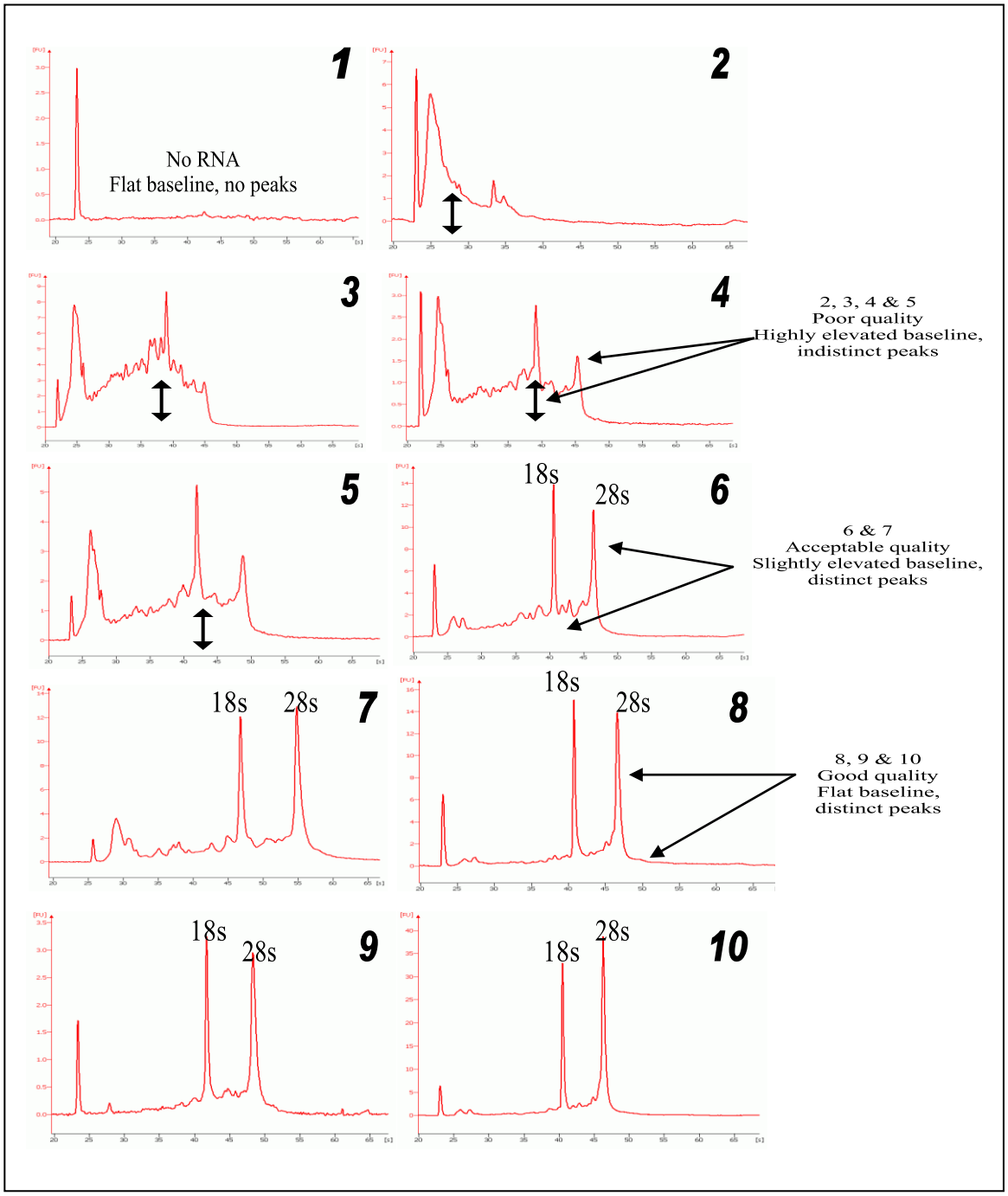


Figure 3.4: Electropherogram images showing different RNA qualities. 1, *contains no RNA and is presented as a flat line with no peaks.* ***2-5,*** *shows poor quality degraded RNA shown by the vertical arrow indicating an elevated baseline with several indistinct peaks.* ***6 and 7*** *are acceptable RNA samples with distinct peaks and slightly raised baseline.* ***8-10*** *represent good quality RNA with varying degrees of height between the 18S and 28S peaks (adapted from Mueller et al., 2004).*

3.4. RESULTS

3.4.1. THE EFFECT OF DIFFERENT BEADS AND MACHINE SETTINGS ON THE DISRUPTION AND HOMOGENISATION OF MASSETER MUSCLE BIOPSIES

Generally, the lysing matrix D® showed better RNA quality than the lysing matrix A and the stainless steel beads. This was similar to the results of Suchak and colleagues (2009) who used the lysing matrix D® and the FastPrep® machine to purify total RNA from fresh human masseter muscle biopsies to be used for real-time PCR gene expression analysis. The lysing matrix D® contained several 1.4mm ceramic spheres which have aided in the disruption and homogenisation, while the lysing matrix A contained one 1/4'' ceramic bead with a garnet matrix. The garnet matrix was a material that is very similar to sand, and even though the lysate solution was spun down prior to transferring to the spin column, traces of the garnet matrix were still present in the lysate solution and partially clogged the silica membrane. This may have resulted in reduced efficiency of the membrane to eliminate DNA contamination. All three stainless steel protocols showed high RNA degradation, which may have been attributed to the heat generated from the stainless steel material while crushing and vibrating.

As for the machine settings, protocol 3 of the lysing matrix D® with two rounds of 20 seconds (speed 6 metres per second) and cooling down for 5 minutes in between rounds was the best protocol. This protocol resulted in an intact RNA in both the lysing matrix D® and A. This may have been attributed to two reasons. First, less time (20s) for the heat to be generated than the 40s protocol. Second, the process of cooling down twice may have prevented RNA degradation. Figure 3.5 demonstrates the Bioanalyser profile and the A260/A280 ratio of the nine different protocols for RNA extraction.

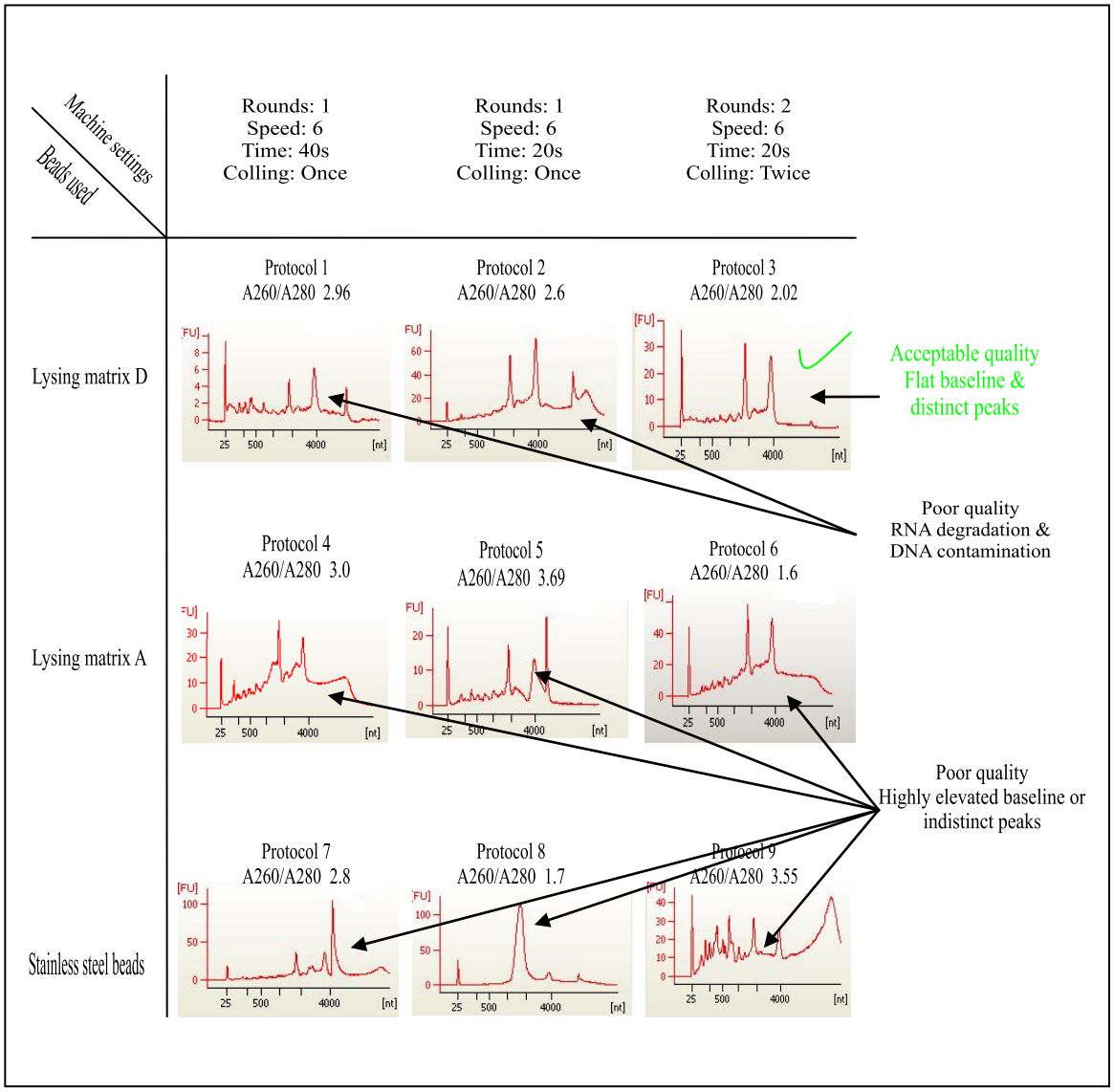


Figure 3.5: Total RNA quality of the nine different protocols. Protocol 1, *using lysing matrix D® with machine settings of 1 round, 40s and cooling down once, has shown low RNA content presented by the small 18S and 28S peaks as well as DNA contamination with a middle peak between both subunits of the rRNA and a high A260/A280 ratio.* ***Protocol 2,*** *using lysing matrix D® with machine settings of 1 round, 20s, and cooling down once, has shown slight RNA degradation presented as an elevated baseline as well as DNA contamination with a large peak at the post-region.* ***Protocol 3,*** *using lysing matrix D® with machine settings of 2 rounds, 20s and cooling twice, has shown good RNA quality with flat baseline and two distinct peaks. However, a small hump was evident at the post-region indicating slight DNA contamination.* ***Protocols 4, 5 and 6*** *of the lysing matrix A with the different machine settings have shown mainly mild-moderate RNA degradation with various baseline elevations and DNA contamination as presented by the peaks at the middle and post regions.* ***Protocols 7, 8 and 9*** *of the stainless steel have shown severe RNA degradation with a single peak only or several indistinct peaks (as in protocol 9).*

3.4.2. THE EFFECT OF DNase DIGESTIONS ON RNA PURIFICATION

The use of the DNase digestion step during total RNA purification produced better RNA quality (with minimal DNA contamination) than the standard RNeasy mini kit procedure (without any DNase digestion). This indicated that, whilst the use of silica membrane spin columns can reduce DNA contamination, skeletal muscles would still require a DNase digestion step to produce a pure RNA sample. Figure 3.6 shows the electropherogram of the three different protocols that were used with and without DNase digestion.

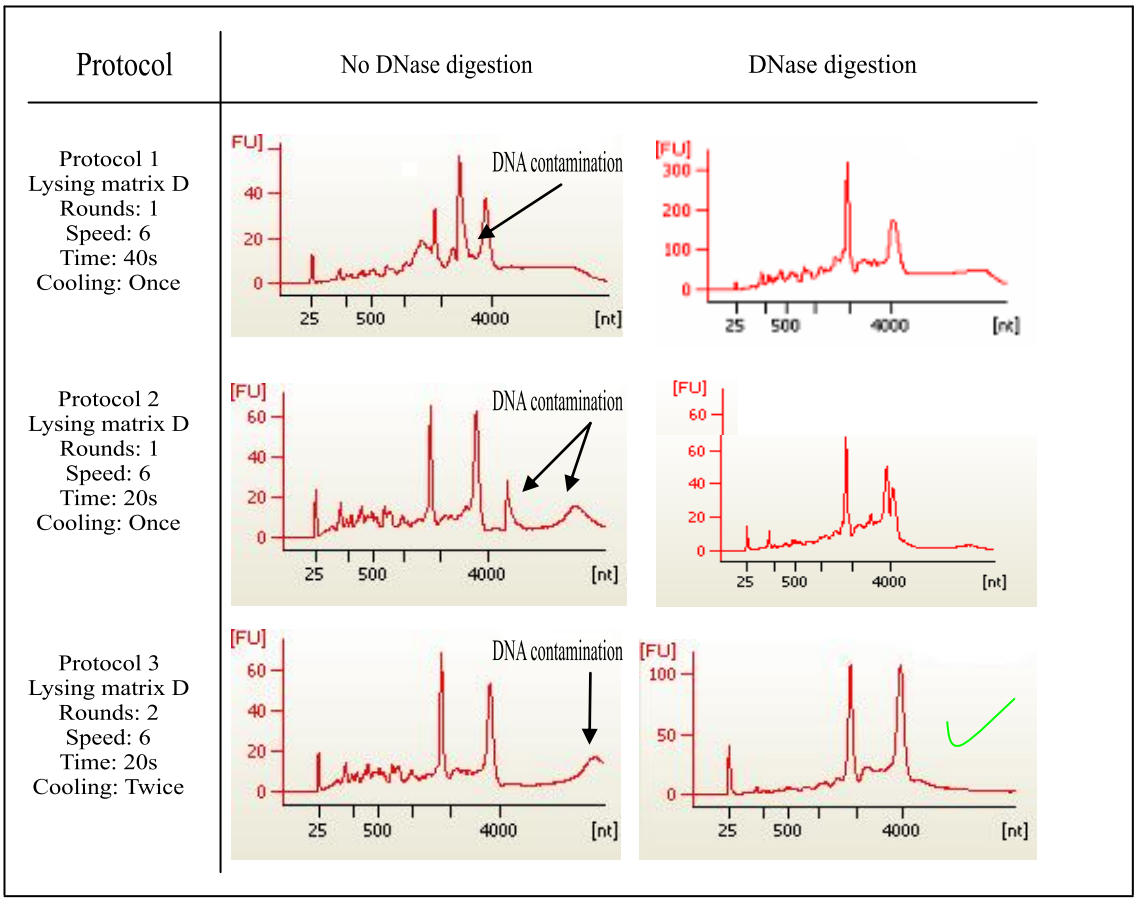
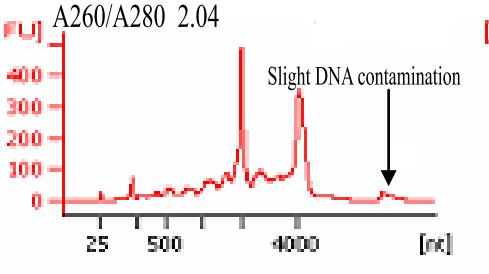


Figure 3.6: Total RNA quality with and without DNase digestion. *All three protocols have shown less DNA contamination when the DNase digestion step was used. DNA contamination was evident in all three protocols without the DNase digestion step. DNA contamination at the mid region (as in protocol 1) and the precursor region (as in protocol 2) were considered critical, while protocol 3 also shows DNA contamination but at the post-region which was not considered critical for gene expression analysis. The RNA degradation that was present in protocol 1 and 2 both with the DNase step and without were related to the machine settings.*

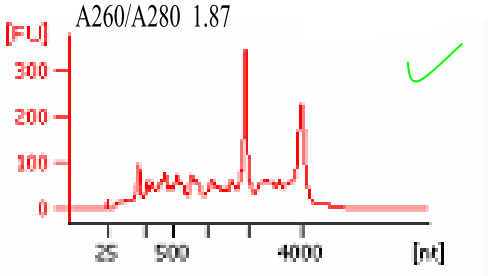
3.5. QUALITY CONTROL OF TOTAL RNA SAMPLES

Total RNA was purified from masseter muscle biopsies of 29 subjects using the lysing matrix D® for disruption and homogenisation, machine settings of 2 rounds, each round for 20 seconds at speed of 6 and cooling down twice for 5 minutes on ice in between rounds. The DNase digestion step on-column was also performed. Generally, all samples exhibited acceptable A260/A280. Most of the samples showed good RNA quality with two distinct peaks, a flat baseline, no DNA contamination or RNA degradation as viewed by the electropherogram image of the Bioanalyser. Other samples were considered with acceptable quality such as samples 1, 8, 9, 16 and 25 which demonstrated a small hump at the post-region indicating a non-critical DNA contamination. Samples 3, 19, 21, 22, 23 and 29 showed reduced 28S peaks which indicated slight RNA degradation, and samples 24 and 26 showed widening of the 18S rRNA which has also indicated slight RNA degradation. The differences in the total RNA quality as viewed by the Bioanalyser profile may have been attributable to due variations in the connective tissue content between individuals, which may have affected disruption and homogenisation and resulted in slight RNA degradation or DNA contamination. However, DNA contamination was seen at the post-region area which according to the manufacturer's guidelines was not considered critical criterion. Therefore, all samples were still considered acceptable as all other electropherogram criteria, as well as the A260/A280 ratios were within the acceptable range (1.68-2.06), and they were therefore included in the gene expression experiments. Figure 3.7 shows the quality control of the 29 total RNA samples.

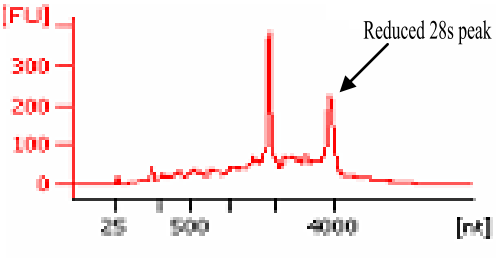
Sample 1
concentration 50ng/ µl
A260/A280 2.04



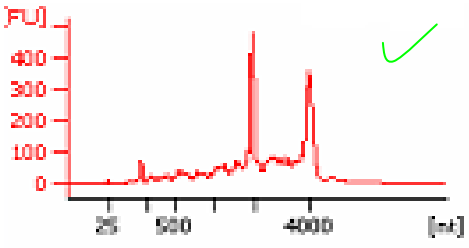
Sample 2
concentration 87ng/ µl
A260/A280 1.87



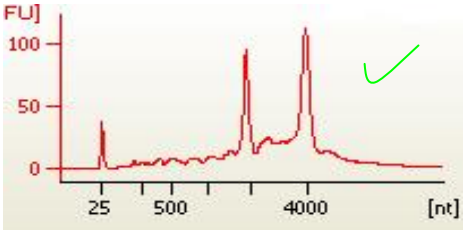
Sample 3
concentration 77ng/ µl
A260/A280 2.01



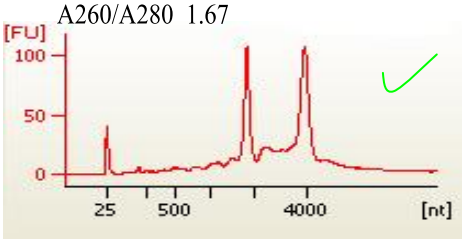
Sample 4
concentration 80ng/ µl
A260/A280 1.83



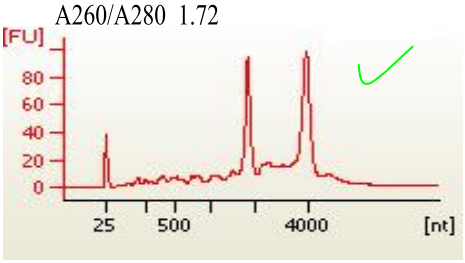
Sample 5
concentration 38ng/ µl
A260/A280 1.69



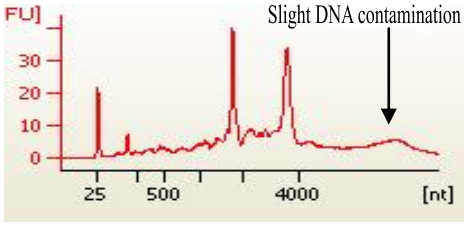
Sample 6
concentration 125ng/ µl
A260/A280 1.67



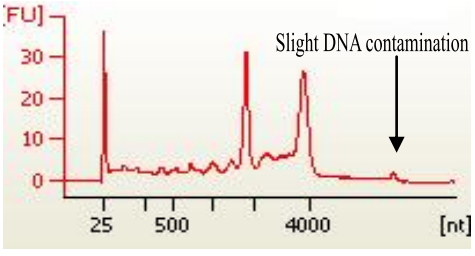
Sample 7
concentration 107ng/ µl
A260/A280 1.72



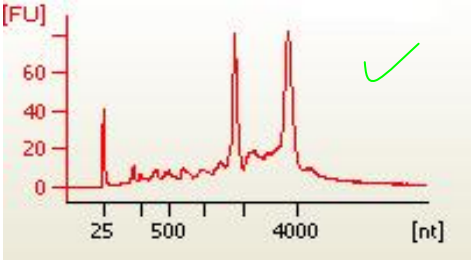
Sample 8
concentration 41ng/ µl
A260/A280 1.68



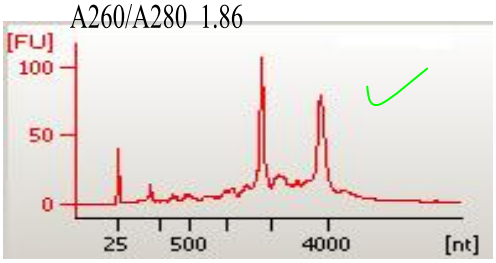
Sample 9
concentration 49ng/ µl
A260/A280 1.8



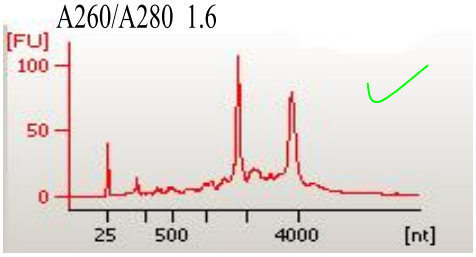
Sample 10
concentration 85ng/ µl
A260/A280 1.91



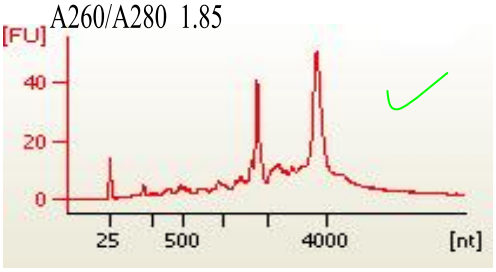
Sample 11
concentration 48ng/ µl
A260/A280 1.86



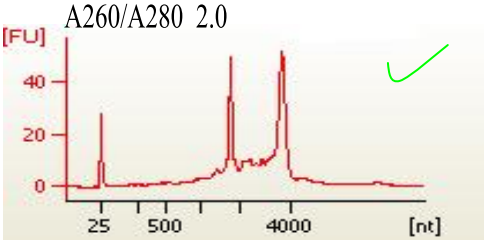
Sample 12
concentration 50ng/ µl
A260/A280 1.6



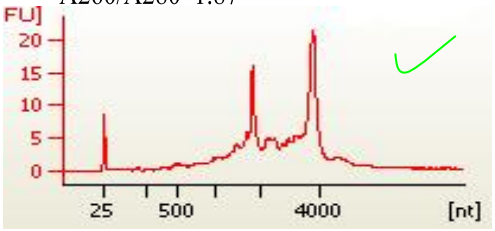
Sample 13
concentration 25ng/ µl
A260/A280 1.85



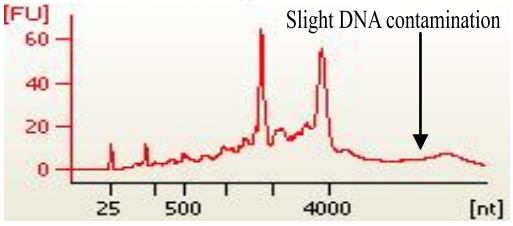
Sample 14
concentration 125ng/ µl
A260/A280 2.0



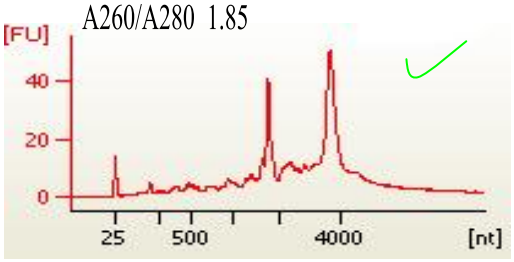
Sample 15
concentration 65ng/ µl
A260/A280 1.87



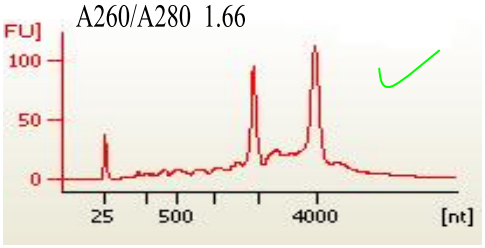
Sample 16
concentration 70ng/ µl
A260/A280 1.79



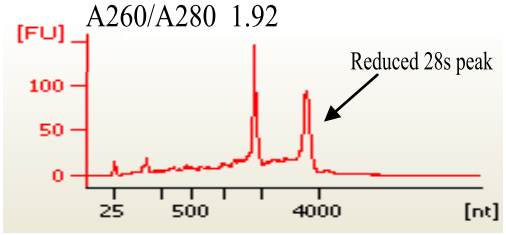
Sample 17
concentration 45ng/ µl
A260/A280 1.85



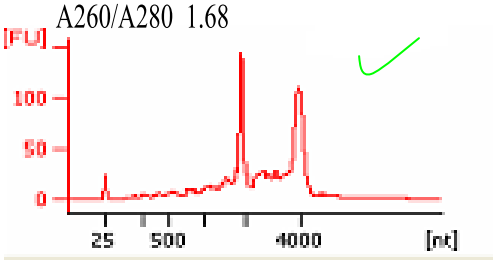
Sample 18
concentration 30ng/ µl
A260/A280 1.66



Sample 19
concentration 89ng/ µl
A260/A280 1.92



Sample 20
concentration 25ng/ µl
A260/A280 1.68



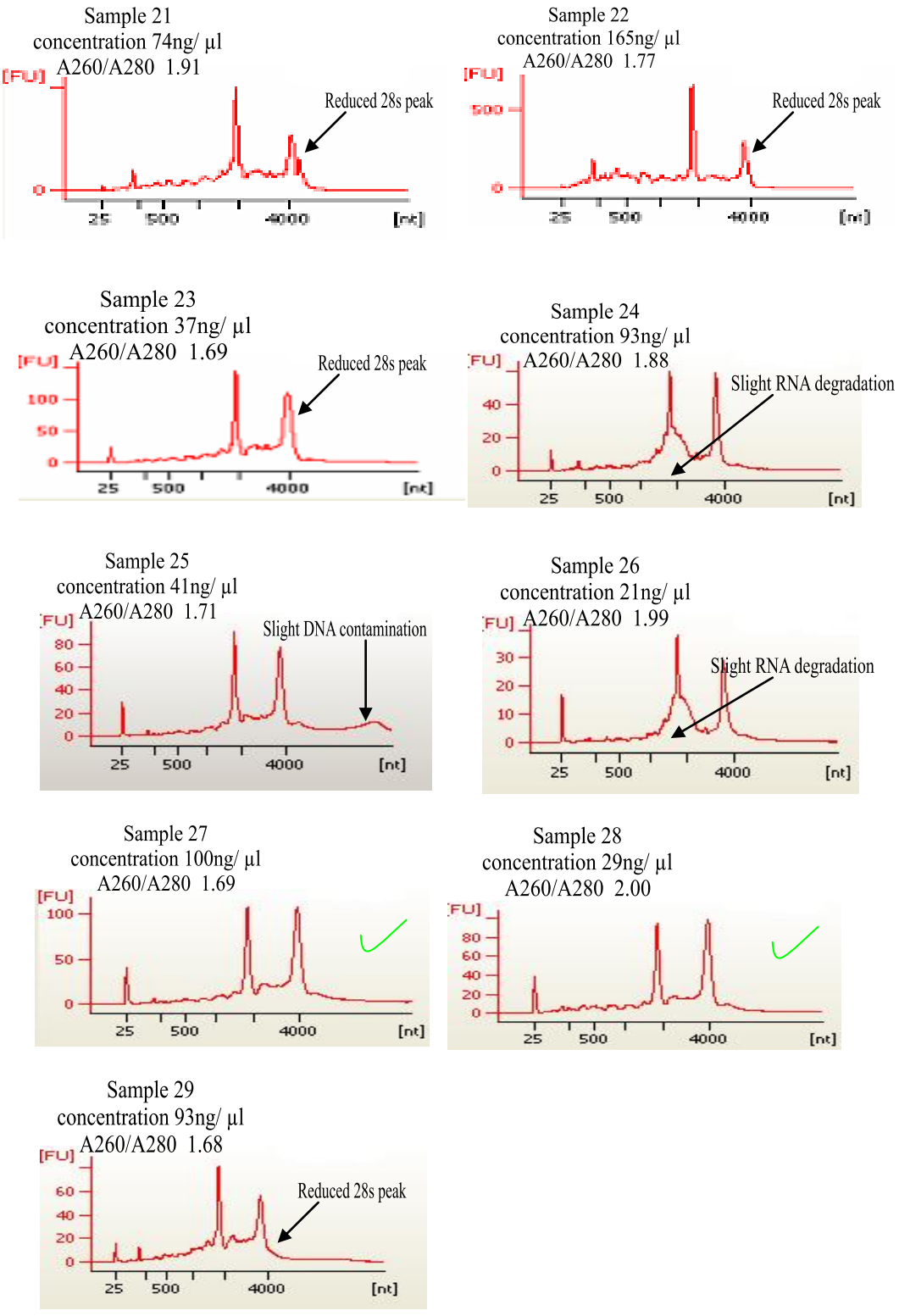


Figure 3.7: Quality control of all 29 total RNA samples included in the current research. *All samples marked with a green tick were of good quality showing acceptable A260/A280 ratio, distinct 18S and 28S rRNA, flat baseline, no RNA degradation or DNA contamination. The remaining samples were of acceptable quality where samples 1, 8, 9, 16 and 25 had slight DNA contamination at the pos-region area which was not considered critical. Samples 3, 19, 21, 22, 23 and 29 showed reduced 28S peak, while samples 24 and 26 demonstrated slight widening of the 18S, all of which may have indicated a slight RNA degradation. All samples were accepted for gene expression experiments.*

3.6. SUMMARY AND CONCLUSIONS

An optimised protocol has been developed to disrupt and homogenise fresh human masseter muscle biopsies using the lysing matrix D®, the FastPrep® machine with 20 seconds, speed 6, 5 minutes cool down on ice, which was repeated twice. A DNase digestion step was incorporated for 15 minutes during total RNA purification and silica membrane spin columns were used for elusion. The protocol has proven its efficiency with good purity, quality and integrity of 29 total RNA samples as shown by the A260/A280 ratios and the Bioanalyser profiles.

Chapter 4. Discovery of masseter muscle candidate genes in relation to non-syndromic craniofacial deformities: A microarray analysis

4.1. INTRODUCTION

Microarray gene expression has been used successfully to identify novel candidate genes in relation to various craniofacial syndromic phenotypes such as clefts (Park et al., 2006), craniosynostoses (Carinci et al., 2002) and hemifacial microsomia (Cai et al., 2005). However, this technique has not been used previously to unravel masseter muscle candidate genes in relation to various non-syndromic craniofacial deformities.

The present research has managed to fully hybridise a total of 27 U133 Plus 2.0 array GeneChips® (including the technical replicate) (materials and methods are presented in Chapter 2, Section 2.7.5). This was using the total RNA purified from fresh human masseter muscle biopsies of 11 control and 15 deformity patients (details of RNA extraction and quality control are presented in Chapter 3). The aim of this chapter is therefore, to present the results of the various analytical steps of the GeneChip® data that have generated the differentially expressed gene lists between vertically and horizontally classified craniofacial groups.

4.2. AFFYMETRIX® GeneChip® QUALITY CONTROL

Following hybridisation of all U133 Plus 2.0 array chips, a GeneChip® quality control step was conducted to ensure efficient labelling and hybridisation procedures as well as proper scanning prior to data pre-processing. This has included seven quality control measures: 1) Poly-A controls; 2) 3'/5' ratio of both ß-actin and GAPDH endogenous reference genes; 3) Values of the hybridisation controls; 4) Percentage of genes expressed; 5) Background values; 6) Raw noise; 7) Scaling factor.

Both technical replicate samples were used during the GeneChip® quality control (QC) and pre-processing data. However, during the microarray statistical analysis, only the 26 samples were included. Samples were numbered from 1 to 27 starting with the 1st batch going through to the 4th batch samples. Both samples 1 and 2 were the technical replicates (**R**) and had comparable results. Out of the 27 samples, 25 passed the seven QC measures, while the remaining two samples (number 10 and 15) failed only the 3'/5' ratio of both control genes. Although samples number 7, 8, 16, 19, 20, 22 and 23 had a higher ß-actin 3'/5' ratio than the Affymetrix recommendations, the GAPDH ratio was acceptable. However, all samples were included in the pre-processing procedure to

further assess the correlation between samples and to identify any outliers. Table 4.1 summarises the general GeneChip® quality control results, while details of the quality control values for each sample Appendix D.

Table 4.1: GeneChip® quality control measures.

Quality control measure	Standard good quality	GeneChip® QC of the current project
Assessment of technical errors (Poly-A control genes) Five prokaryotic genes (dap, lys, phe, thr) were spiked-in during sample preparation. Any technical errors would prevent the expression of these genes.	Similar expression of the five genes between different array samples.	-All 27 samples passed.
Assessment of RNA quality and labelling (3'/5' ratio) Both GAPDH and ß-actin are long genes and if RNA degradation occurs it usually starts at the 5' end, while labelling occurs from the 3' end. Poor RNA quality or inefficient labelling would produce large variations in the expression between 3' and 5' probes resulting in a high ratio.	Ratio < 3 for both GAPDH and ß-actin genes.	-25 samples passed, including both technical replicates. -2 samples failed both genes (samples 10 and 15). -7 samples failed the ß-actin gene (samples 7, 8, 16, 19, 20, 22 and 23).
Assessment of the labelling process (hybridisation controls) Four prokaryotic control genes (bioB, bioC, bioD and cre) were spiked-in during hybridisation and are prepared in different concentrations. They should have increasing intensities reflecting their increasing concentrations.	Increasing intensity call starting from bioB < bioC < bioD < cre.	-All 27 samples passed
Assessment of technical replicates (% of genes expressed) The number of probe-sets expressed relative to the total number of probe-sets.	Technical replicates should have less than 10% difference	-Both technical replicates had a difference of 4.9% (passed).
Assessment of the effect of the scanner (Background values) Arrays scanned with the same scanner should have comparable background values.	No specific Affymetrix guidelines. Typical range 20-100	-All 27 samples had comparable background values (passed).

Quality control measure	Standard good quality	GeneChip® QC of the current project
Raw noise values Noise is the pixel-pixel variation of probes on the scanned array chip. Both the scanner and sample quality can affect this value.	Comparable values between chips.	-All 27 samples had comparable raw noise value (passed).
Scaling factors In arrays containing large number of transcripts it is expected that some of the genes will be expressed differently between samples, while the majority would not change. The aim of scaling is to produce similar average intensities of the unchanged genes across all arrays. A broad range of scaling factor would indicate variable RNA quality or quantity between samples.	Affymetrix recommend a range of scaling factor less than 3 fold-change (< 3 SD)	-All 27 samples had a scaling factor less than 3 (passed).
All 27 samples passed 6 quality control measures. As for the 7th measure which was the 3'/5' ratio, 2 samples failed the 3'/5' ratio of both genes and the other 7 samples failed the 3'/5' ratio of ß-actin gene only.		

4.3. PRE-PROCESSING OF DATA AND NORMALISATION

Pre-processing of data included normalisation and removal of any systematic variations between arrays. The data were viewed in different formats of plots, histograms and charts to assess and exclude any poorly correlated chips. Sample number 15 was the least correlated sample and showed large variations in relation to all other samples. Although this sample showed a good Bioanalyser profile when the total RNA was assessed in the previous Chapter 3 (Figure 3.7, sample number 15), partial RNA degradation might have occurred during the laboratory procedure. Table 4.2 summarises the results obtained from the different plots and histograms. All graphs, plots, histograms and charts are presented in Appendix D.

Table 4.2: Description of normalisation graphs used.

Normalisation graph	Standard good quality	Normalisation results for the current project
Box plots Used to identify outliers in microarray normalised data.	Normalised box plots of all samples should have comparable medians.	-No obvious outliers were detected (passed).

Normalisation graph	Standard good quality	Normalisation results for the current project
Perfect-match (PM) histogram This histogram represents a measurement of the density of the probe intensities of the PM probes. Used also to identify outliers.	Normalised date would show density skewed to the right with 2 distinct peaks. All samples should have similar histogram appearance.	-Typical histogram appearance. -No obvious outliers were detected (passed).
PM vs. MM histogram The mis-match (MM) probes measure non-specific hybridisation while PM probes measure specific hybridisation. It is expected that the PM probes will produce strong intensities while the MM probes weaker intensities.	Normalised data would show differences between the PM and MM curves.	-All samples had typical histogram appearance (passed).
RNA degradation plot The probe-sets for each control gene were designed with 11 probes (0-10). The 0 probe presenting the most 5' sequence and the 10th probe is the most 3' sequence. Since RNA degradation starts at the 5' end, and labelling occurs from the 3' end, therefore it is expected that the probes at the 3' have higher intensity than the probes at the 5'.	Intensities of the genes were expected to be higher towards the 3' probes.	-All samples showed typical RNA degradation plot appearance (passed).
MvA plots A graphical representation of general gene expression variation between two samples only within the same classified group. The fold-change (M) at the y-axis is plotted against the average fluorescence intensity (A) of both samples at the x-axis.	If both samples are highly comparable they will have a symmetric appearance around the x-axis (leaf like appearance).	-Sample 8 and 15 showed large variations when compared to other samples within the same classified group.

Normalisation graph	Standard good quality	Normalisation results for the current project
Correlation plot This is a heatmap of the chip-chip Spearman's rank correlation coefficients. This plot can detect outliers.	Arrays of technical replicates should have a correlation coefficient near to 1.0. Other chips also should have high correlation coefficient near (0.97-1.0)	-Both technical replicates had a high correlation around 0.98. -Sample 8 showed acceptable correlation to other samples with a correlation coefficient of almost 0.95 -Sample 15 showed the least correlation coefficient of almost 0.90.
All samples showed comparable normalised data except for sample 15 that was the least correlated sample.		

4.4. DIFFERENTIALLY EXPRESSED GENE LISTS

Sample number 15 (Class II long face) failed the 3'/5' ratio during quality control, it was the least correlated sample when viewing the MvA plots and the correlation heatmap and showed large variations in relation to other samples. Therefore, it was excluded from further analysis.

A total of 25 normalised GCRMA files (excluding the technical replicate sample) were analysed twice to generate two separate differentially expressed gene lists. First, a gene list for the vertically classified groups, and a second for the horizontal craniofacial groups. These classifications were based on simple craniofacial criteria similar to previous masseter muscle gene expression studies (Nelson-Moon et al., 1998; Gedrange et al., 2005 and 2006; Harzer et al., 2007; Suchak et al., 2009). All significant genes had a p-value of ≤ 0.001.

4.4.1. DIFFERENTIALLY EXPRESSED GENE LIST OF THE LONG FACE

The first analysis produced a differentially expressed gene list between 11 long face patients vs. 14 controls. Out of 38,500 array genes, 19 genes were found to be down-regulated and 12 up-regulated in long face patients compared to the controls (total of 31 genes) (Table 4.3).

Table 4.3: Differentially expressed gene list for the long face pattern.

Affy probe-set ID	Gene symbol	Chromosome	Fold change
Long face vs. Ctrl			
Down-regulated genes	19 genes		
209829_at	C6ORF32	6	-9.75
213645_at	ENOSF1	18	-4.32
207864_at	SCN7A	2	-2.79
1554549_a_at	WDR20	14	-2.36
243829_at	BRAF	7	-2.23
201797_s_at	VARS	6	-2.03
208024_s_at	DGCR6	22	-1.78
1558942_at	ZNF813	19	-1.64
225525_at	KIAA1671	22	-1.64
1557567_a_at	LOC148987	1	-1.62
216497_at	LOC120364	11	-1.62
217365_at	PRAMEF5	1	-1.51
213445_at	ZC3H3	8	-1.44
221924_at	ZMIZ2	7	-1.25
219521_at	B3GAT1	11	-1.23
1554451_s_at	DNAJC14	12	-1.18
217647_at	HP	16	-1.18
206459_s_at	WNT2B	1	-1.14
241934_at	HNT	11	-1.08
Up-regulated genes	12 genes		
214357_at	C1ORF105	1	29.2
221950_at	EMX2	10	9.85
229414_at	PITPNC1	17	7.89
1568658_at	LOC339804	2	5.7
207563_s_at	OGT	X	2.6
244377_at	SLC1A4	2	2.31
223082_at	SH3KBP1	X	2.04
1554168_a_at	SH3KBP1	X	1.95
222876_s_at	CENTA2	17	1.64
242647_at	USP34	2	1.55
225124_at	PPP1R9B	17	1.4
232767_at	CADM1	11	1.4
Total	31 genes		

Genes were arranged based on the heights of fold change. Ctrl: Control. _at: probe-sets that binds to anti-sense targets. _s: probe-sets that have multiple transcripts shared between different genes. _a: probe-sets that can recognize an alternative splice variant of a gene.

4.4.2. DIFFERENTIALLY EXPRESSED GENE LISTS OF HORIZONTAL DEFORMITIES

The second analysis generated four differentially expressed gene lists comparing different horizontal groups. The groups included 11 controls, 4 Class II and 10 Class III patients. Out of 38,500 genes, a total of 85 genes were found to be differentially expressed between the various horizontal deformities. Table 4.4 presents details of both the up- and down-regulated genes from the four gene lists.

Table 4.4: Differentially expressed gene lists for Class II and Class III horizontal patterns.

Affy probe-set ID	Gene symbol	Chromosome	Fold change
1. Class II vs. Ctrl			
Down-regulated genes	9 genes		
221950_at	EMX2	10	-24.1
229414_at	PITPNC1	17	-13.5
1568658_at	LOC339804	2	-9.45
1553071_a_at	MYOZ3	5	-9.13
213555_at	RWDD2	6	-5.31
1553070_a_at	MYOZ3	5	-3.92
1554168_a_at	SH3KBP1	X	-3.27
223082_at	SH3KBP1	X	-2.77
218472_s_at	PELO	5	-2.25
Up-regulated genes	3 genes		
1556462_a_at	LOC730245	13	3.41
212240_s_at	PIK3R1	5	3.18
209829_at	C6ORF32	6	1.92
Total	12 genes		
2. Class III vs. Ctrl			
Down-regulated genes	21 genes		
210794_s_at	MEG3	14	-22.0
236977_at	LOC646588	7	-21.7
229414_at	PITPNC1	17	-12.9
204472_at	GEM	8	-11.1
203649_s_at	PLA2G2A	1	-10.7
221950_at	EMX2	10	-9.78
206404_at	FGF9	13	-8.22
210444_at	NPY6R	5	-6.19
201609_x_at	ICMT	1	-5.78
221922_at	GPSM2	1	-3.61

Affy probe-set ID	Gene symbol	Chromosome	Fold change
213555_at	RWDD2	6	-2.97
232531_at	EMX2OS	10	-2.95
218678_at	NES	1	-2.75
219747_at	C4ORF31	4	-2.5
232983_s_at	SERGEF	11	-2.48
223082_at	SH3KBP1	X	-2.28
212627_s_at	EXOSC7	3	-2.25
218780_at	HOOK2	19	-2.17
1554168_a_at	SH3KBP1	X	-2.14
224523_s_at	C3ORF26	3	-2.10
209452_s_at	VTI1B	14	-1.71
Up-regulated genes	15 genes		
224209_s_at	GDA	9	25.8
205007_s_at	CIB2	15	7.84
209829_at	C6ORF32	6	6.77
208148_at	*MYH*4	17	4.38
215629_s_at	KIAA1799	1	4.2
241764_at	LOC284825	21	3.81
1560750_at	LOC151121	2	2.87
1556462_a_at	LOC730245	13	2.33
204347_at	LOC645619	12	2.19
215262_at	OXNAD1	3	2.16
1554549_a_at	WDR20	14	2.13
201797_s_at	VARS	6	1.73
244791_at	PHFDHL1	13	1.62
217365_at	PRAMEF5	1	1.55
209563_x_at	CALM1	14	1.48
Total	36 genes		
3. Class II vs. III			
Down-regulated genes	7 genes		
227556_at	ATP1B1	1	-3.94
203364_s_at	KIAA0652	11	-2.62
214279_s_at	NDRG2	14	-2.58
1555278_a_at	CKAP5	11	-1.83
1568955_at	SRGAP2	1	-1.76
224836_at	TP53INP2	20	-1.73
229474_at	MICAL3	22	-1.67
Up-regulated genes	5 genes		
229250_at	TPCN2	11	2.77

Affy probe-set ID	Gene symbol	Chromosome	Fold change
1555439_at	GTF3C3	2	2.51
240451_at	HIRA	22	1.87
228271_at	SND1	7	1.84
220575_at	FAM106B	17	1.44
Total	12 genes		
4. All vs. Ctrl			
Down-regulated genes	19 genes		
236977_at	LOC646588	7	-28.1
210794_s_at	MEG3	14	-24.9
221950_at	EMX2	10	-15.3
207992_s_at	AMPD3	11	-15.2
229414_at	PITPNC1	17	-13.2
201609_x_at	ICMT	1	-6.63
239860_at	NPY6R	5	-6.59
1568658_at	LOC339804	2	-5.7
213555_at	RWDD2	6	-3.97
203151_at	MAP1A	15	-3.63
203695_s_at	DFNA5	7	-3.61
1553070_a_at	MYOZ3	5	-2.73
1554168_a_at	SH3KBP1	X	-2.66
232983_s_at	SERGEF	11	-2.64
219747_at	C4ORF31	4	-2.62
223082_at	SH3KBP1	X	-2.51
224523_s_at	C3ORF26	3	-2.45
218472_s_at	PELO	5	-2.1
209452_s_at	VTI1B	14	-1.7
Up-regulated genes	6 genes		
209829_at	C6ORF32	6	11.4
1556462_a_at	LOC730245	13	2.81
212240_s_at	PIK3R1	5	2.46
201797_s_at	VARS	6	1.91
244791_at	PHGDHL1	13	1.75
209563_x_at	CALM1	14	1.58
Total	25 genes		

Genes were arranged based on the heights of fold change. Ctrl: Control. _x: probe-sets where it was not possible to design them with a unique sequence to the corresponding gene and can cross-hybridise.

4.4.3. FILTERING THE DATA

The forward stepwise logistic regression analysis was conducted twice. The first analysis included the GCRMA values of the 31 genes reported in the vertical gene list. Out of the 31 differentially expressed genes, two genes (KIAA1671 and DGCR6) were found to be significantly different and down-regulated in long face patients compared to the controls. The 85 genes of the horizontal groups, on the other hand, were included in the second analysis which revealed three significantly different genes. One gene (NDRG2) was found to be down-regulated in Class II patients compared to Class III individuals, while Class III samples showed down-regulation of one gene (SERGEF) compared to the controls. Another gene (LOC730245) was up-regulated in both Class II and Class III patients compared to the controls. All five genes had a highly significant p-value of ≤ 0.001. Table 4.5 lists the five significantly different genes.

Table 4.5: Final candidate gene list for both vertical and horizontal deformities.

Patient 's group	Gene name	Symbol	FC	Ch Location	Microarray gene expression status
Vertical groups	2 genes				
Long vs. Ctrl	1) KIAA1671	KIAA1671	-1.64	22q11.23	Down-R long face
	2) DiGeorge syndrome Critical Region gene family member 6	DGCR6	-1.78	22q11.21	Down-R long face
Horizontal groups	3 genes				
Class II vs. III	1) N-myc Down Regulated Gene family member 2	NDRG2	-2.58	14q11.2	Down-R Class II
Class III vs. Ctrl	2) Secretion Regulating Guanine nucleotide Exchange Factor	SERGEF	-2.64	11p14.3	Down-R Class III
Class II and Class III vs. Ctrl	3) Hypothetical protein Locus 730245	LOC730245	2.81	13q22.1	Up-R Class II and Class III

FC: Fold change. Ch: Chromosome. Ctrl: Control. R: Regulated.

4.5. DISCUSSION

The aim of the current microarray analysis was to identify masseter muscle candidate genes in relation to various craniofacial deformities. The initial differentially expressed gene lists that were generated for the long face, Class II and Class III deformities contained many up- and down-regulated genes, any of which can be considered a masseter muscle candidate gene and would warrant further investigation in relation to craniofacial deformities. However, the process of selecting a specific gene from the candidate gene list to be further followed-up is not a standardised procedure.

Novel genes may have been overlooked during the selection procedure, particularly in studies of original design that have not been previously duplicated (Chuaqui et al., 2002). It is for this reason that several authors have implemented logistic regression statistical analyses to filter microarray data and to identify potential genes from the candidate gene list (Wang et al., 2006; Eijssen et al., 2008). The current research has used the forward stepwise logistic regression analysis to filter the gene list and identified 2 novel genes associated with the long face deformity and 3 other genes associated with horizontal deformities, and these will be investigated further.

4.5.1. NOVEL MASSETER MUSCLE GENES IN RELATION TO LONG FACE DEFORMITY

Out of 38,500 genes, a total of 31 genes were found to be differentially expressed between long face patients and controls, 19 of which were down-regulated and 12 up-regulated in long face patients compared to individuals with average vertical facial phenotypes. Out of the 31 candidate genes, statistical analysis revealed 2 genes, namely DGCR6 and KIAA1671, that were down-regulated in the long face pattern compared to the controls.

4.5.1.1. DGCR6 gene

The DGCR6 gene (DiGeorge syndrome Critical Region gene number 6) was first described by Demczuk and colleagues (1996), who observed the presence of this gene in a critical region (22q11.21) with 500 kilo bases (Kb) close to deletion regions at the 22q11 area that were mainly linked to DiGeorge syndrome (Lindsay, 2001), and was associated with mild forms of the condition. Patients with DiGeorge syndrome exhibit a

wide range of clinical phenotypes including palatal malformations (clefts), nasal and ear abnormalities and a small mandible associated with a retrognathic appearance (de Lonlay-Debeney et al., 1997).

Protein activity of the DGCR6 gene has been suggested to play an important role in cell migration and attachment (Demczuk et al., 1996) and was found to be over-expressed in skeletal muscle, as well as heart, liver and cancer cell lines compared to other tissue cells such as brain and pancreas (Pfuhl et al., 2005). Furthermore, the DGCR6 gene has been found to modulate expression of other neighbouring genes, one of which was the TBX-1 gene (Hierck et al., 2004). The TBX-1 (Arnold et al., 2006) and other members of the T-box gene family (Braybrook et al., 2002) have been reported in patients with various types of orofacial clefts.

This may suggest that the DGCR6 gene may recruit different pathways with either a direct effect on the development of the mandible resulting in a retrognathic appearance, or an indirect role, modulating other genes which further affect the development of the maxilla and may result in the formation of a prognathic appearance. Interestingly, the current microarray data showed down-regulation of this gene in relation to both prognathic and retrognathic patients who shared similar long face appearance compared to individuals with average vertical facial features. This may suggest impaired function of this gene not only in relation to horizontal deformities, but also in relation to imbalanced vertical facial development.

4.5.1.2. KIAA1671 gene

Genes with the name KIAA are human genes that encode large proteins with an unidentified function (Suyama et al., 1999). These types of genes were investigated by the Kazusa DNA Research Institute and have been designated the name “KIAA” with four-digit numbers (Kikuno et al., 2004). Large microarray platforms such as Affymetrix® often included uncharacterised genes such as KIAA and hypothetical protein genes to aid in the identification of their function in various tissues (Handrigan et al., 2007). Up to date, no specific function has been identified for the KIAA1671 gene which is located on chromosome 22q11.23 and is in close proximity to the DGCR6 gene (chromosomal location 22q11.21). As discussed earlier, the DGCR6 gene

has been reported to modulate the expression of neighbouring genes which have followed a similar gene expression to DGCR6 (Hierck et al., 2004). The current microarray data showed a down-regulation of the DGCR6 gene in long face patients, a reason that may have affected transcription of the KIAA1671 gene and resulted in under-expression of this gene also in long face individuals.

4.5.2. NOVEL MASSETER MUSCLE GENES IN RELATION TO CLASS II AND CLASS III DEFORMITY

Out of 38,500 genes, a total of 12 genes (9 down-regulated and 3 up-regulated) were associated with a Class II pattern compared to the controls (average horizontal features), while Class III patients had a total of 36 differentially expressed genes (21 down-regulated and 15 up-regulated) compared to the controls. Analysis of the Class II group compared to the Class III pattern revealed a total of 12 differentially expressed genes (7 up-regulated and 5 down-regulated in the Class II pattern). Further, statistical analysis ascertained 3 genes to be further investigated in relation to the various horizontal craniofacial deformities. One gene was found to be down-regulated in Class II individuals compared to Class III patients, namely NDRG2. As for the remaining two genes, one was up-regulated (hypothetical protein LOC730245) in both Class II and Class III patterns compared to the controls, and the other gene (SERGEF) was down-regulated in only Class III patients compared to the controls.

4.5.2.1. NDRG2 gene

The NDRG2 (Neuroblastomas-myelocytomatosis "N-*myc*" Downstream Regulated Gene number 2) has a chromosomal location 14q11.2 and is a member of the NDRG family, which is composed of three other members (Zhang et al., 2006). The expression of the NDRG family is believed to be involved in cell proliferation and differentiation (Knoepfler et al., 2002). The NDRG2 gene has been found to be highly expressed in adult skeletal muscles than other NDRG members (Qu et al., 2002) and has been found to be down-regulated by high levels of N-*myc* gene, while low levels of N-*myc* have resulted in over-expression of the NDRG2 gene (Zhang et al., 2006).

The current microarray results indicate a down-regulation of the NDRG2 gene in Class II patients compared to Class III individuals. This may suggest that under-expression of

the NDRG2 gene is associated with a retrognathic phenotype, while over-expression may be related to a prognathic appearance. However, a complex cascade with other regulatory genes such as N-*myc* is suspected to be involved.

4.5.2.2. Hypothetical protein LOC730245 gene

The hypothetical proteins are genetic transcripts with unknown function which are often included in microarray gene chips to discover their role in particular tissues (Suravajhala, 2007). The hypothetical protein locus (LOC) 730245 was located on the 13q22.1 area. No specific function has been previously identified for the LOC730245. However, interstitial deletions at the 13q22.1 region have been associated with Hirschprung's disease (Lamont et al., 1989). Some forms of this disease were accompanied by general growth retardation, narrow forehead, restricted upper dental arch, protruded upper incisors, cleft palate and general muscle weakness (Amiel and Lyonnet, 2001). The present study's microarray data showed an over-expression of the LOC730245 gene in both Class II and Class III patterns compared to the controls, with the Class II group having a higher fold change (3.41) than Class III patients (2.33).

A combination of long face pattern with both Class II and Class III deformities was reported with weak masseter muscle activity compared to individuals with average vertical and horizontal facial morphology (Cha et al., 2007). Class II patients recruited for the current study were long face patients, while the Class III group exhibited both long and average vertical facial features. This may explain the higher fold change of the LOC730245 in Class II patients than Class III individuals. Furthermore, this may suggest that over-expression of the LOC730245 gene may be associated with weak masseter muscle activity of patients with combined vertical and horizontal craniofacial deformities.

4.5.2.3. SERGEF gene

The SERGEF gene (Secretion Regulating Guanine-nucleotide Exchange Factor), was first described and characterised at a chromosomal location 11p14.3 which was critical (in close proximity) to a locus associated with various hereditary severe congenital deafness disorders (Uhlmann et al., 1999). Furthermore, the amino acid sequence of the SERGEF protein showed high sequence homology to a large protein super family called

the guanine nucleotide exchange factor. Therefore the SERGEF gene was initially called Deafness Locus associated putative Guanine-nucleotide Exchange Factor (DelGEF). Under-expression of the DelGEF protein has been found to increase the secretion, but not the synthesis, of the chondroitin sulphate proteoglycans in cancer cell lines and was therefore called Secretion Regulating Guanine-nucleotide Exchange factor (SERGEF) (Sjölinder et al., 2002).

The synthesis of chondroitin sulphate proteoglycan was found to be increased during adult muscular regeneration following injury (Carrino et al., 1988). This indicates a major role of the chondroitin sulphate proteoglycans during muscle regeneration. The present microarray data showed a down-regulation of the SERGEF in patients with a Class III prognathic appearance which may lead to the over-expression of chondroitin sulphate proteoglycans in the extracellular matrix of the masseter muscle, suggesting a need in the masseter muscles of such patients for continuous repair and regeneration.

No definite conclusion can be drawn from microarray data without further testing. This is due to the nature of microarray platforms, where thousands of genes and hypotheses are tested in one experiment, and it is expected that false positive results may occur (Shi et al., 2006). Therefore, the gene expression of the 5 novel genes needs to be tested against the clinical, dental and radiographic criteria of patients with various craniofacial discrepancies using quantitative RT-PCR, which is a more sensitive technique (data are presented in Chapter 6).

4.6. SUMMARY AND CONCLUSION

- To the best of my knowledge, this has been the first study to ascertain masseter muscle candidate genes in relation to non-syndromic craniofacial deformities using microarray technology.

- A total of 25 samples were analysed twice to produce candidate gene lists in relation to both vertical and horizontal craniofacial phenotypes. Further statistical analysis of initial candidate gene lists has pointed out 5 novel genes not previously reported in relation to the masseter muscle of non-syndromic individuals exhibiting various craniofacial deformities.

- Out of the 5 novel genes, two were related to the long face pattern (DGCR6 and KIAA1671), while three other genes (NDRG2, LOC730245 and SERGEF) were differentially expressed in relation to horizontal deformities.

- Although the DGCR6 gene has been down-regulated in long face patients compared to the controls, this gene may be involved in the development of both vertical and horizontal craniofacial phenotypes.

- The KIAA1671 gene which lies in a chromosomal location close to the DGCR6 gene was also down-regulated in long face patients compared to the controls. The DGCR6 gene was reported to modulate the expression of neighbouring genes which follow similar expression to DGCR6. Therefore, it is possible to hypothesise that the expression status of the KIAA1671 gene may have been modulated by the DGCR6 gene.

- The NDRG2 gene was down-regulated in Class II patients compared to Class III individuals. However, the NDRG2 gene has been known to be regulated by the N-*myc* gene. Therefore, a complicated mechanism is suspected with various other genes affecting the NDRG2 expression in the masseter muscle of patients with a prognathic and a retrognathic appearance.

- The LOC730245 gene was up-regulated in both Class II and Class III patients compared to the controls. Over-expression of the LOC730245 gene has been suggested to be related to weak masseter muscles of patients with various vertical and horizontal craniofacial deformities.

- The SERGEF gene was down-regulated in Class III patients compared to the controls. Under-expression of SERGEF gene has been known to increase the secretion of the chondroitin sulphate proteoglycan which is involved in regenerative processes. Hence, it is suspected that the masseter muscle of patients with a prognathic appearance is subject to continuous micro trauma which may necessitate continued regeneration.

Chapter 5. Difficulties of patient's classification for phenotype-genotype studies

5.1. INTRODUCTION

The main aim of a phenotype-genotype study is to relate a specific clinical scenario to a particular genetic background, whether at genomic (Braybrook et al., 2001), transcriptomic (Suchak et al., 2009) or proteomic level (Tippett et al., 2008). However, craniofacial deformities exhibit a wide range of clinical phenotypes which can be found in both syndromic (Whitaker et al., 1981; Kimonis et al., 2007) and non-syndromic (Singh and Bartlett, 2005) individuals, showing varying degrees of severity (Renier et al., 2000).

To overcome this challenge, clinicians have critically analysed craniofacial morphology to identify basic, combined and comprehensive descriptive features which were significantly different between average individuals and patients with craniofacial deformities (Sassouni, 1969; Jacobson et al., 1974; Schendel et al., 1976). For example, a Class III pattern describes single dimensional criteria, while a Class III long face explains two dimensional features. Comprehensive description, on the other hand, provides details of affected skeletal and dental components, such as Class III horizontal deformity with a retruded maxilla, protruded mandible and long face vertically with dental anterior open bite.

Previous genetic studies of the masseter muscle have investigated single dimensional discrepancies looking at either vertical or horizontal features of recruited subjects. However, it is more frequent to see a patient with a combination of both vertical and horizontal patterns rather than a single dimensional discrepancy (Sassouni, 1969; Proffit and White, 1990). It was, therefore, not known whether grouping patients based on a single craniofacial phenotype is sufficient for genetic analysis.

The previous chapter demonstrated the use of basic types of craniofacial classifications (vertical classification -long face and average vertical face; horizontal classification - Class II, Class III and average horizontal face) with the microarray technology. However, a detailed analysis of the clinical, dental and skeletal components of the craniofacial deformities is needed to produce a better correlation between phenotypic appearance and genotypic background. The aims of this chapter are therefore to:

- Briefly review the literature to identify the various classifications and subdivisions of basic craniofacial patterns.

- Highlight the types of classifications that have been used previously in masseter muscle genotype-phenotype research.

- Identify the clinical, dental and skeletal criteria of the subjects for the current research and assess the feasibility of implementing these criteria into different phenotypic classifications to be further used in relation to qRT-PCR genetic data.

5.2. CLASSIFICATION OF CRANIOFACIAL PATTERNS

5.2.1. BASIC CLASSIFICATION OF CRANIOFACIAL PATTERNS

Sassouni (1969) identified four basic craniofacial patterns based on either vertical or horizontal skeletal discrepancies. These basic patterns were described in Chapter 1 as the long and short faces of the vertical dimension and Class II and Class III in the horizontal plane.

5.2.2. COMBINED CLASSIFICATION OF CRANIOFACIAL PATTERNS

A more detailed classification would include a combination of both vertical and horizontal patterns (Sassouni, 1969), more often seen clinically than single dimensional discrepancies (Proffit and White, 1990). For example, Class II or Class III facial horizontal patterns can be associated with average, long or short vertical dimensions.

5.2.3. COMPREHENSIVE CLASSIFICATION OF CRANIOFACIAL PATTERNS

A comprehensive classification would include skeletal and dental subdivisions of the basic facial pattern. The long face (Schendel et al., 1976), short face (Opdebeeck and Bell, 1978), Class II (Antonini et al., 2005) and Class III (Jacobson et al., 1974) patterns have all been reported with various subdivisions and varying degrees of maxillary, mandibular and dental discrepancies.

5.2.3.1. Subdivisions of the long face pattern

Depending on the mandibular ramus height (RH), the mandibular angulation and the presence or absence of an anterior open bite (AOB), two subdivisions have been reported with the long face pattern. One division was observed with a short RH, increased mandibular angulation and AOB, while the other variant was reported with an increased RH, average mandibular angulation and without AOB (Schendel et al., 1976; Fields et al., 1984). Figure 5.1 shows examples of radiographic tracings of the two types of the long face pattern.

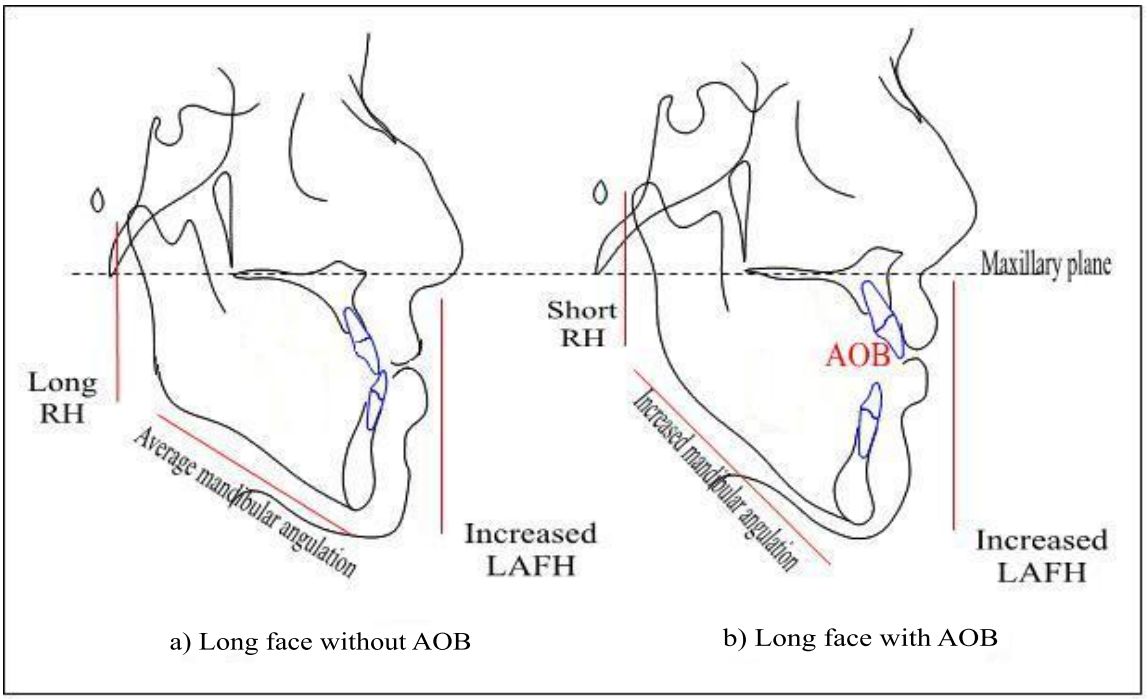


Figure 5.1: Subdivisions of the long face pattern. *Both groups show increased lower anterior face height (LAFH).* ***a)*** *Long face patient with increased ramus height (RH), average mandibular angulation and no anterior open bite (AOB).* ***b)*** *Long face individual with short RH, increased mandibular angulation and AOB.*

5.2.3.2. Subdivisions of the short face pattern

Two subdivisions have also been reported with the short face pattern, by which the posterior vertical maxillary length, ramus height and the relationship of the mandibular plane to the cranial base were all different between subdivisions. One type of the short face had a normal posterior vertical maxillary height, long ramus and a sharply reduced mandibular plane to the cranial base angle. The other subgroup exhibited a short ramus, marked deficiency in the posterior maxillary height leading to a closing rotation of the

mandible, a deep bite and a reduced mandibular plane angle in relation to the cranial base (Opdebeeck and Bell, 1978). Figure 5.2 shows examples of radiographic tracings of the two subdivisions of the short face pattern.

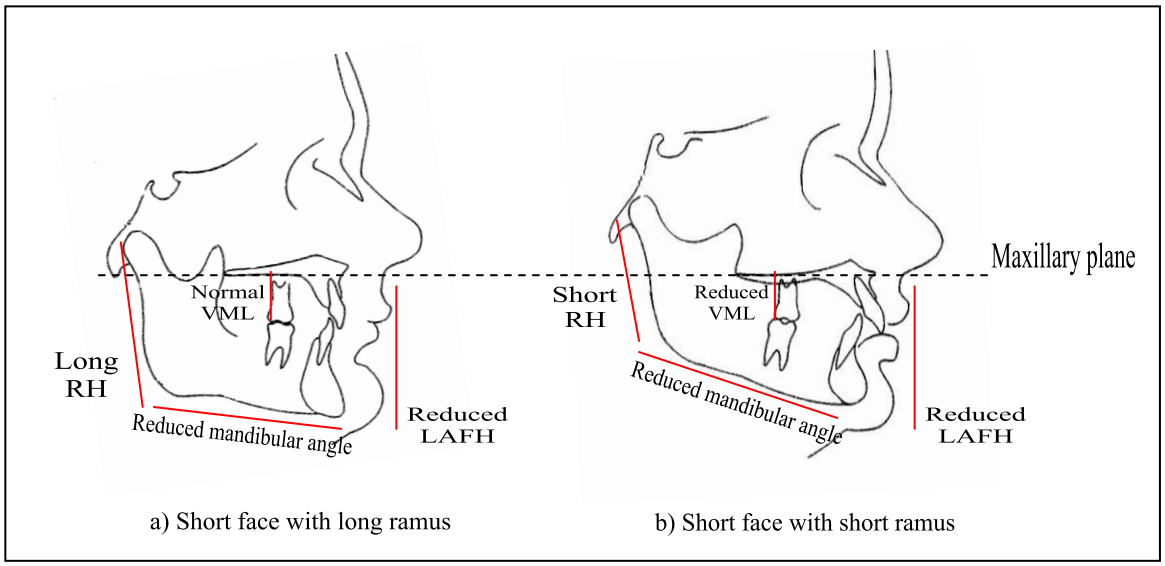


Figure 5.2: Subdivisions of the short face pattern. *Both groups show reduced LAFH and mandibular angulation.* ***a)*** *Short face patient with increased ramus height (RH) and normal vertical maxillary length (VML).* ***b)*** *Short face individual with short RH and reduced VML. This diagram was adapted and modified from Opdebeeck and Bell (1978).*

5.2.3.3. Subdivisions of Class III pattern

Several authors (Björk, 1955; Sanborn, 1955; Jacobson et al., 1974) have identified different types of skeletal Class III depending on the affected skeletal component, whether it be the maxilla, the mandible or both. Four main subgroups of skeletal Class III have been reported in the literature (Figure 5.3):

1. Normal maxilla and prognathic mandible: This subgroup is one of the most common types seen in non-syndromic individuals (Sanborn, 1955; Lew and Foong, 1993). However, this type has also been reported in male patients with aneuploidal abnormalities in the x-chromosome (XXY -Klinefelter syndrome) where mandibular growth is increased (Gorlin et al., 1965).

2. Normal mandible and retruded maxilla: This type is associated with various cleft anomalies (Hayashi et al., 1976), some of the craniosynostotic conditions

(Costaras-Volarich and Pruzansky, 1984; Langford et al., 2003) as well as non-syndromic individuals (Dietrich, 1970).

3. Combination of retrusive maxilla and prognathic mandible: This type is also common in non-syndromic individuals (Ellis and McNamara, 1984).

4. Maxilla and mandible within the normal range of prognathism: This type was described as the maxilla being at the lowest average range of SNA° and the mandible at the highest average range of the SNB°. It is the least common subgroup of the Class III pattern (Sanborn, 1955).

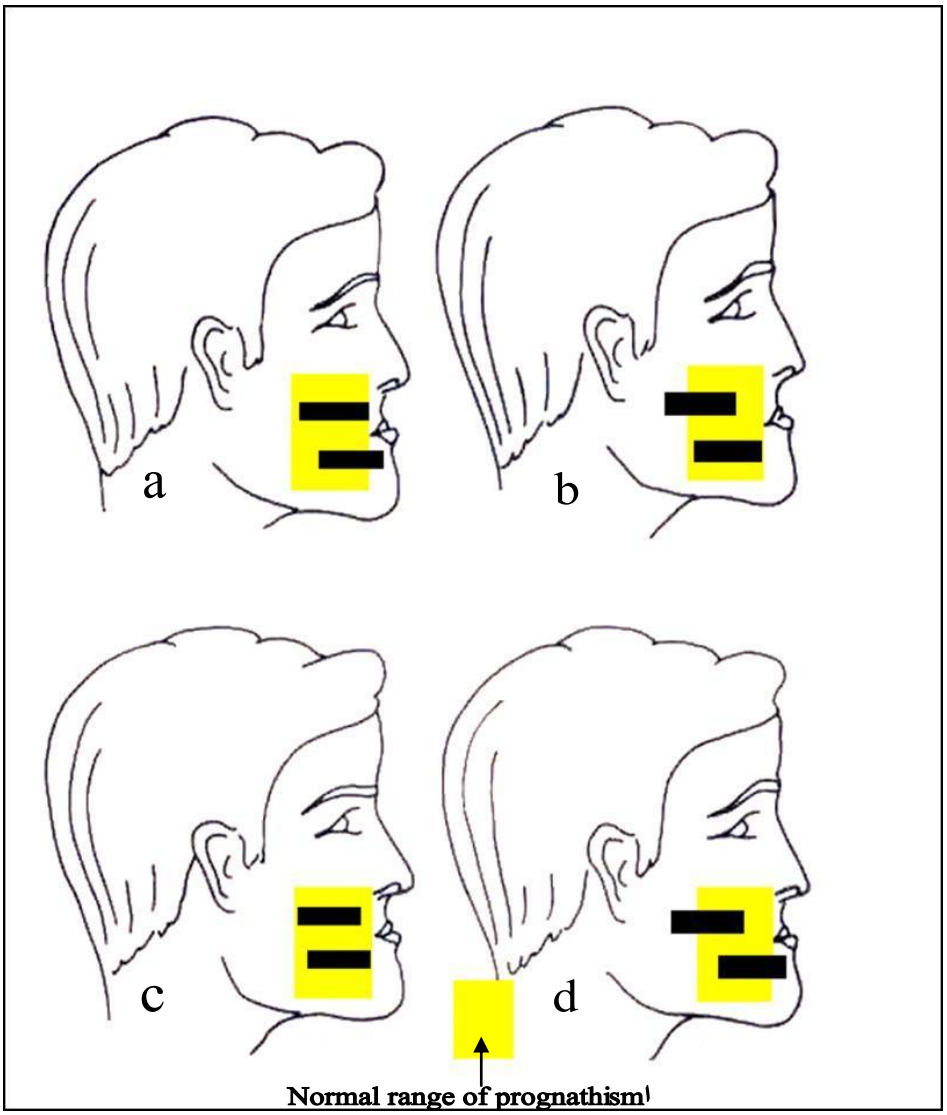


Figure 5.3: Subdivisions of the Class III pattern. *The normal range of prognathism marked in yellow was identified as the normal range of both the SNA° (dictates the position of the maxilla) and the SNB° (dictates the position of the mandible) (Jacobson et al., 1974).* ***a)*** *Normal maxilla and prognathic mandible.* ***b)*** *Normal mandible and retruded maxilla.* ***c)*** *Maxilla and mandible within the normal range of prognathism.* ***d)*** *Combination of retrusive maxilla and prognathic mandible. This diagram was adapted from Jacobson et al., 1974.*

5.2.3.4. Subdivisions of Class II pattern

Similar to the Class III pattern, different types of Class II retrognathic skeletal appearance have been reported in the literature. Maxillary protrusion and normal mandibular growth (Antonini et al., 2005), a normal maxilla and an underdeveloped or retruded position of the mandible (Ngan et al., 1997; Sayin and Turkkahraman, 2005) and a protruded maxilla with a retruded mandible (Akemi, 2005) are all variations that have been reported in both syndromic (e.g. XO -Turner syndrome with deficient growth of the mandible) (Gorlin et al., 1965) and non-syndromic (de Freitas et al., 2005) individuals. However, the normal maxilla and underdeveloped mandible is the most common type of the Class II phenotype (Sidlauskas et al., 2006).

Figure 5.4 illustrates the different subdivisions of the vertical and horizontal craniofacial patterns.

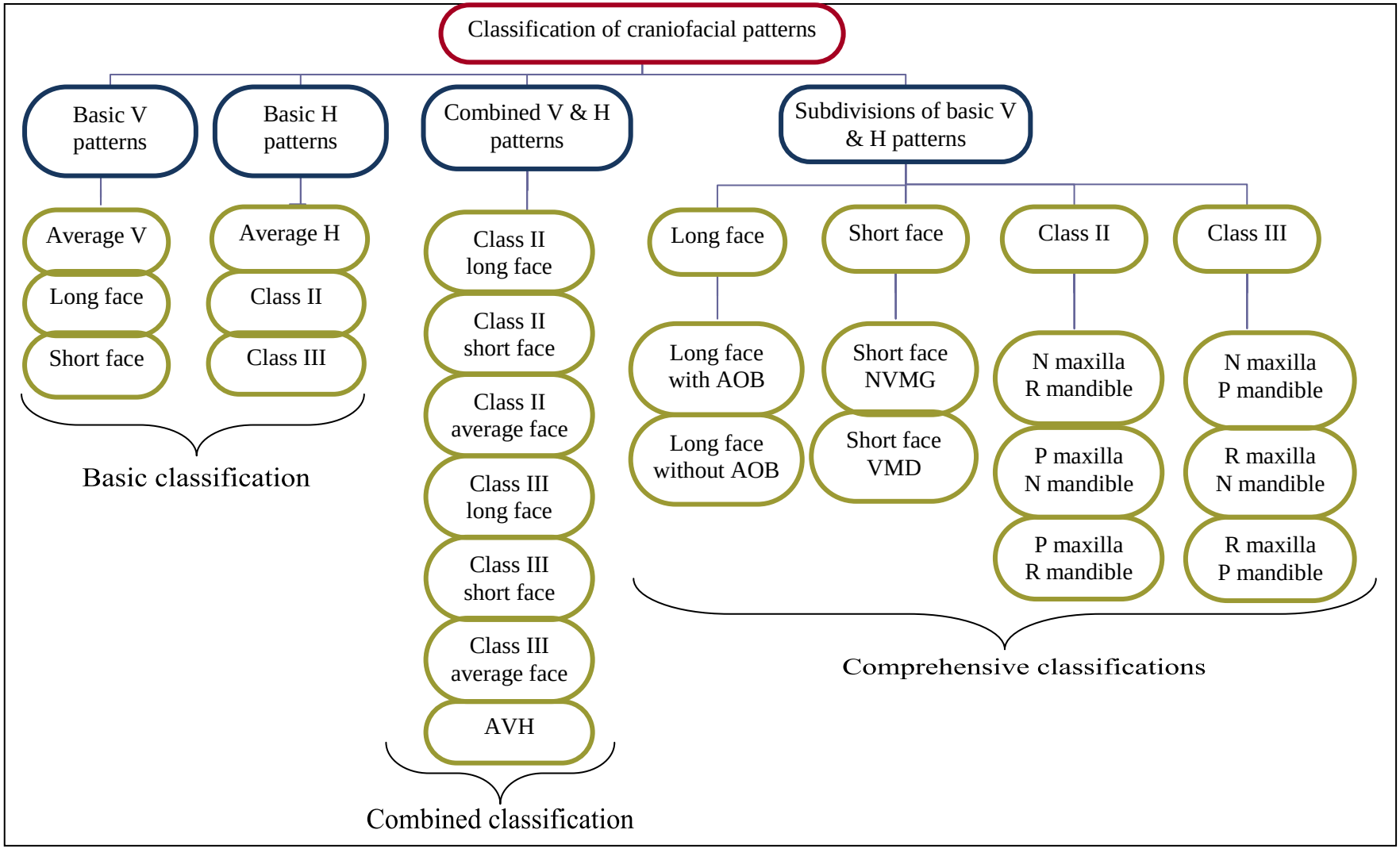


Figure 5.4: The various subdivisions of the vertical and horizontal facial patterns. *V: Vertical. H: Horizontal. AOB: Anterior open bite. AVH: Average vertical and horizontal. NVMG: Normal vertical maxillary growth. VMD: Vertical maxillary deficiency. N: Normal. R: Retruded. P: protruded.*

5.3. CLASSIFICATIONS USED IN PREVIOUS MASSETER MUSCLE GENOTYPE-PHENOTYPE STUDIES

Previous masseter muscle genotype-phenotype studies have looked at either vertical (mainly long face) (Nelson-Moon et al., 1998; Price et al., 1998; Singh et al., 2000; Tippett et al., 2008; Suchak et al., 2009) or horizontal dimensional discrepancies (Class II and Class III) (Gedrange et al., 2005; 2006; Harzer et al., 2007; Maricic et al., 2008). These studies have mainly used basic vertical and horizontal types of classifications.

5.3.1. STUDIES ASSESSING VERTICAL FACIAL DEVELOPMENT

Nelson-Moon and colleagues (1998) in a study of six myosin heavy chain (*MYH*) genes, recruited 15 patients, all undergoing orthognathic surgery, 9 of whom were long face. The remaining 6 patients exhibited horizontal facial deformities but with average vertical facial features and were considered as controls. Similarly, Tippett and co-workers (2008) recruited 20 subjects, 10 of whom were identified clinically and radiographically as long face subjects, while the other 10 controls were patients receiving surgical correction of horizontal deformities and were identified with average vertical facial features. Other investigators have used a "weighted-points system" based on various vertical cephalometric parameters to group patients into either long face or average controls (Price et al., 1998; Singh et al., 2000). Most of these studies have included patients with horizontal deformities having average vertical facial features as controls. As such it is likely that both Class II long face and Class III long face patterns were combined as one single deformity group.

5.3.2. STUDIES ASSESSING HORIZONTAL FACIAL DEVELOPMENT

Horizontal assessment of patients who were classified for masseter muscle genotype-phenotype analysis have been based mainly on clinical appearance, radiographic ANB angle and the dental overjet, with patients classified into either Class II or Class III regardless of the vertical facial pattern (Gedrange et al., 2005; 2006). 10 deformity patients (5 Class II and 5 Class III individuals) were recruited but there were no controls. A recent study with a larger sample size (30 subjects -16 Class II and 14 Class III) selected patients with pure horizontal deformities, using the SN-MP angle to exclude patients with vertical discrepancies (Harzer et al., 2007).

None of the previous masseter muscle genotype-phenotype studies, whether vertical or horizontal, have included patients with average vertical and horizontal facial features as controls. Furthermore, the effect of various subdivisions of Class II, Class III and long face patterns on masseter muscle gene expression analysis has not been previously tested. The following sections will identify the clinical, dental and radiographic criteria of the 29 subjects in the present study to assess the feasibility of implementing their criteria into various phenotypic classifications to be further analysed in relation to qRT-PCR masseter muscle gene expression.

5.4. MATERIALS AND METHODS

The following sections will briefly describe the materials and methods that were used to assess the clinical, dental and radiographic criteria of the 29 subjects.

5.4.1. CLINICAL ASSESSMENT

Clinical assessment of both vertical and horizontal patterns was conducted based on the previously described criteria in Chapter 2 (Section 2.7.8.1). The clinical assessment was used for simple and combined pattern grouping, including average control (vertically and horizontally), long face, short face, Class II and Class III patterns.

5.4.2. DENTAL ASSESSMENT

- Overbite

Overbite was assessed clinically using a dental ruler and was measured in millimetres. The dental assessment was used to identify the various vertical subgroups including AOB or deep bite.

- The number of dental occlusal contacts

The number of dental occlusal contacts has been found to affect masseter muscle gene expression (Nelson-Moon et al., 1998; Harzer et al., 2007) and was therefore recorded for the current research and used to assess whether these were different between various craniofacial groups. The pre-treatment study models were trimmed to match the occlusion of the 29 subjects and were used to record the static occlusal contacts using pink wax blocks. By adjusting the trimmed margins of both the upper and lower casts on a bench, to simulate the occlusion of the patient, the wax was heated and pressed

against both casts and an imprint of the occlusion was recorded on the wax. This was followed by counting the number single contacts imprinted on the wax.

5.4.3. RADIOGRAPHIC ASSESSMENT

- Vertical variables

Both the LAFH% and TAFH were used as general parameters to identify vertical facial pattern (normal mean value ± 1 SD –control; above norm –long face; below norm – short face), while overbite, TPFH, RH and SN-MP angle were used to identify the various vertical subgroups (as described by Schendel et al., 1976; Opdebeeck and Bell, 1978).

- Horizontal variables

The ANB angle was used to identify the general horizontal pattern (normal mean value ± 1 SD –control; above norm –Class II; below norm –Class III). Both the SNA and SNB angles were used to assess the position of the maxilla and the mandible, respectively, in relation to the cranial base (normal mean value ± 1 SD –normal; above norm – protruded; below norm –retruded). All cephalometric variables and cephalometric Caucasian norms are described in details in Chapter 2 (Section 2.4.4.3).

5.4.4. STATISTICAL ANALYSIS

5.4.4.1. Data analysis of radiographic variables

- Bland and Altman's approach (described in Chapter 2, Section 2.3.6.4.2) was used to assess the reliability and reproducibility of the cephalometric variables using repeated measurements of 25 cephalometric radiographs.

- To assess the significance of variation in vertical and horizontal cephalometric variables between the different classified groups, both the Mann Whiney U-test (two groups) and Kruskal Wallis test (more than two groups) were conducted using SPSS v14 (statistical package of social sciences). A p-value of ≤ 0.05 was considered to be statistically significant. The graphical representation of cephalometric variations between groups was generated using SPSS v14 in the form of box plots.

5.4.4.2. Data analysis of the number of dental occlusal contacts

To assess the significance of differences in the number of occlusal contacts between the different classified groups, both the Mann Whiney U-test (two groups) and Kruskal Wallis test (more than two groups) was conducted using SPSS v14. A p-value of ≤ 0.05 was considered to be statistically significant. The graphical representation of the variations in the number of occlusal contacts between groups was generated using SPSS v14 in the form of box plots.

5.5. RESULTS

5.5.1. RELIABILITY AND REPRODUCIBILITY OF RADIOGRAPHIC MEASUREMENT

Bland and Altman's approach revealed good correlation and agreement between repeated measurements as shown by the Lin's concordance correlation coefficient which were close to 1. The lowest value was 0.95 which was related to the LAFH%. The mean difference between paired measurements was less than 0.5 in all radiographic variables, while the paired t-tests revealed no significant differences between paired measurements. Thus minimal systematic errors were encountered.

As for random errors, the LAFH, RH and SNA angle showed the highest coefficient of repeatability and a wide range of the limits of agreement, indicating difficulty in repeating these measurements. This may have been attributed to the poor quality of some of the radiographs which made it difficult to assess the N-point (affected the LAFH), the A-point (affected the SNA), and the Go- and Ar-points (affected RH). These anatomical landmarks have been reported to be more difficult to locate on cephalometric radiographs and are less reliable than others (Sandler, 1988). However, this did not affect the classification, as most of the subjects had severe deformities with extreme radiographic measurements compared to the controls. Table 5.1 represents values obtained by using Bland and Altman's approach, while Figures 5.5 and 5.6 represent the horizontal and vertical Bland and Altman's graphs.

Table 5.1: Reliability and reproducibility of radiographic variables using the Bland and Altman's approach.

Radiographic parameter	**Lin's Concordance Correlation Coefficient**	**Bland and Altman's approach**				
		Systematic error		*Random error*		*Limits of agreement*
		Mean difference	paired t-test p-value	SDD	CR	
SNA^{o}	0.98	-0.40	0.09	1.15	2.25	-2.7-1.9
SNB^{o}	0.99	-0.20	0.23	0.82	1.60	-1.8-1.4
ANB^{o}	0.99	-0.26	0.09	0.74	1.45	-1.7-1.2
LAFH%	0.95	0.20	0.12	0.64	1.26	-1.1-1.5
TAFH mm	0.98	-0.46	0.26	1.99	3.90	-4.4-3.4
TPFH mm	0.98	0.32	0.29	1.50	2.90	-2.6-3.3
RH mm	0.97	0.16	0.60	1.54	3.02	-2.9-3.2
SN-MP^{o}	0.98	0.24	0.30	1.30	2.55	-2.3-2.8

All radiographic variables had good correlation and agreement between repeated measurements as shown by the Lin's concordance correlation coefficient. Minimal systematic errors were detected as evident by the non significant p-values of the paired t-test and low mean differences between repeated measurements. The TAFH, RH and the SNA measurements were difficult to repeat as shown by the high coefficient of repeatability (CR). SDD: Standard deviation of differences.

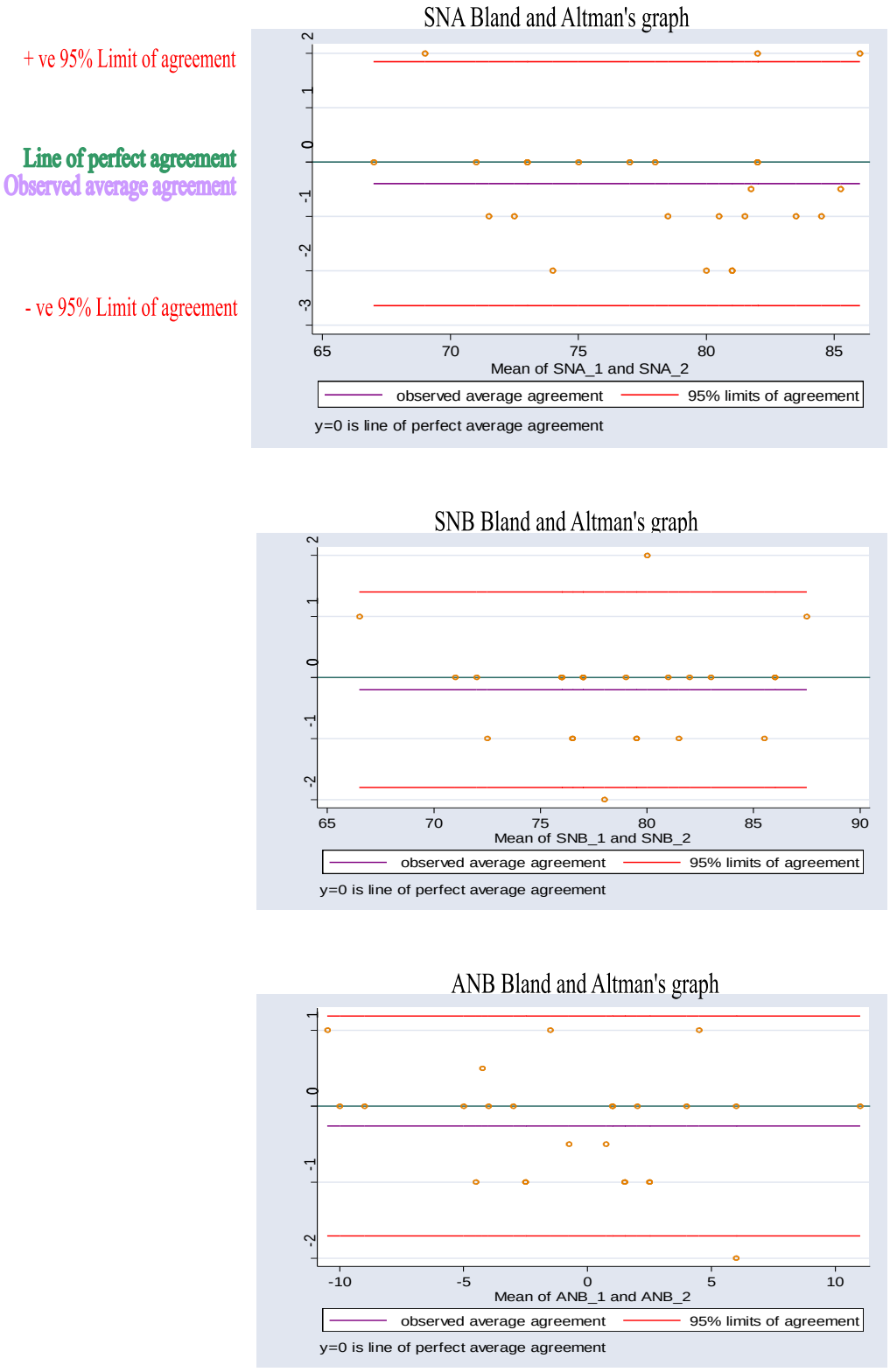


Figure 5.5: Bland and Altman's graphs for horizontal cephalometric variables. *Generally, all horizontal variables showed low systematic errors as indicated by the close proximity of the observed horizontal line in relation to the zero horizontal line which represents the perfect agreement. The SNA angle showed higher limits of agreement (-2.7-1.9), as indicated by the two horizontal red lines, than the SNB (-1.8-1.4) and the ANB angle (-1.7-1.2), which indicates higher random error of the SNA angle.*

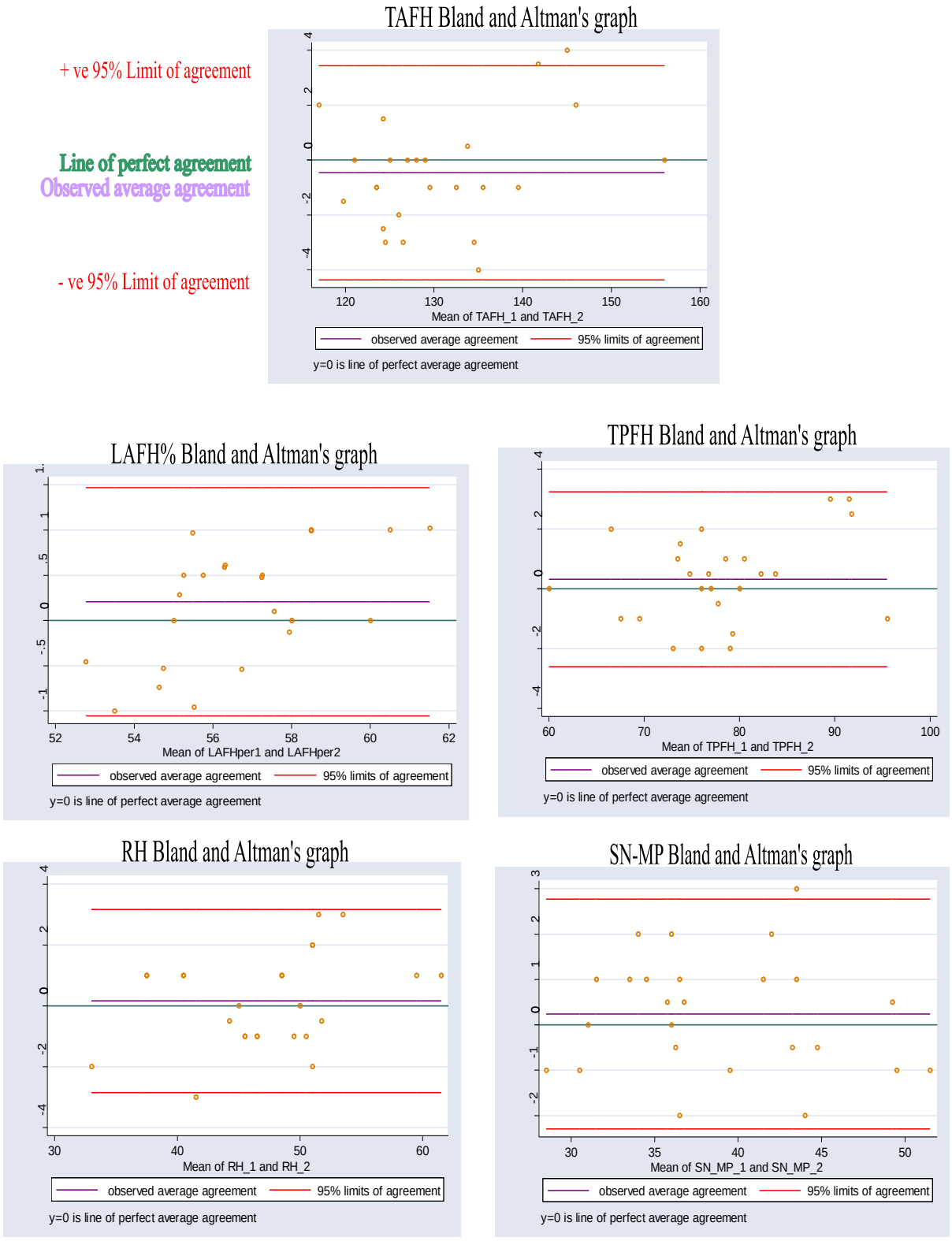


Figure 5.6: Bland and Altman's graphs for vertical cephalometric variables. *Generally, all vertical variables showed low systematic errors as indicated by the close proximity of the observed horizontal line in relation to the zero perfect agreement line. The LAFH showed the highest limits of agreement (-4.4-3.4) with higher random errors, compared to the lowest limits of agreement of the LAFH% (-1.1-1.5).*

5.5.2. CLINICAL, DENTAL AND RADIOGRAPHIC ASSESSMENT OF THE SUBJECTS

Following the clinical, dental and radiographic assessment of the 29 subjects, it was found they exhibited variable clinical and radiographic combinations of dental and skeletal components both in the vertical and horizontal dimensions. Furthermore, the 29 subjects could be grouped in different ways based on the previously discussed basic, combined (Sassouni 1969) and comprehensive classifications and subdivisions (Jacobson et al., 1974; Schendel et al., 1976; Opdebeeck and Bell, 1978). Figure 5.7 shows how patients move from one group to another depending on the investigated dimensional discrepancy and classification implemented.

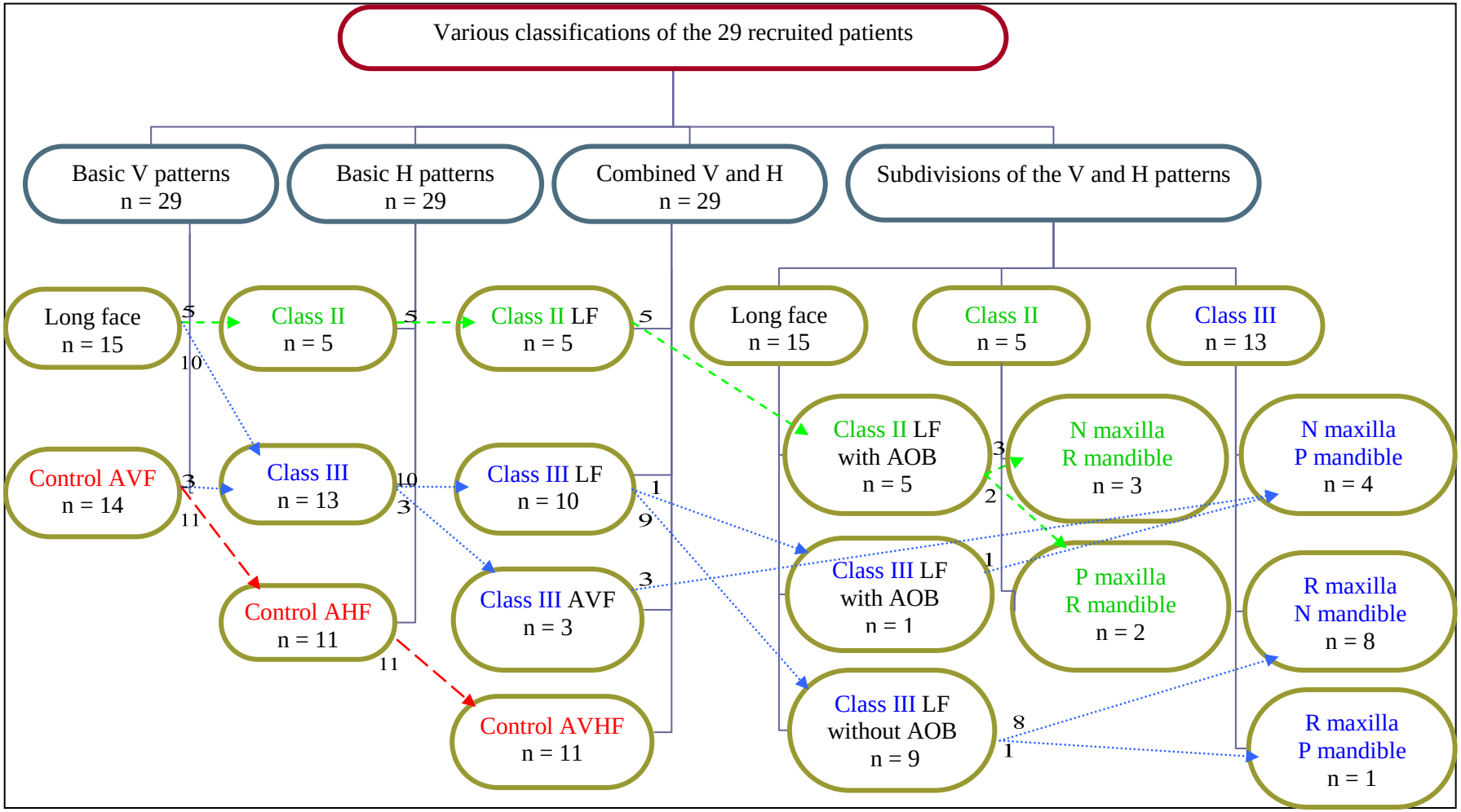


Figure 5.7: Clinical, dental and radiographic criteria of the recruited patients described through various classifications. *The arrows indicate how various groups of patients shifted from one group to another based on the classification (e.g. a group of Class III patients with average vertical facial features were grouped as controls in the basic vertical classification and as deformity when using the basic horizontal classification). V: Vertical. H: Horizontal. LF: Long face. AVF: Average vertical face. AHF: Average horizontal face. AVHF: Average vertical and horizontal face. AOB: Anterior open bite ≥ 3mm. N: Normal. R: Retruded. P: Protruded.*

Classification of subjects for a phenotype-genotype study is clearly difficult, as encountered in previous studies, because of the low numbers of subjects in each subdivision compared to the wide range of clinical, dental and radiographic components. However, it was felt useful to consider the basic vertical and horizontal classifications to allow comparison to previous masseter muscle studies (Harzer et al., 2007; Suchak et al., 2009), as well as studying the combined vertical and horizontal classification and subdivisions of the long face pattern (with and without AOB). This was done to assess whether different classifications of the same subjects could have an effect on the genetic analysis. Furthermore, the Class II, Class III and long face patterns exhibited varying degrees of maxillary, mandibular and dental severity. Therefore, it was appropriate to use non-parametric correlation analysis (Spearman's correlation coefficient analysis), where all skeletal cephalometric and dental variations could be tested separately against masseter muscle gene expression (details of the phenotype-genotype correlation analysis are presented in Chapter 6).

5.5.3. PHENOTYPIC CLASSIFICATIONS GENERATED

5.5.3.1. Basic vertical classification

The 29 subjects were classified on their vertical facial appearance regardless of any horizontal deformities. This classification generated two groups; 14 control and 15 long face patients. None of the subjects exhibited a short face appearance. This classification was similar to the vertical facial grouping that was used to analyse microarray data (Chapter 2, Section 2.7.8.1).

Table 5.2 summarises the clinical, radiographic and dental variations between the long face pattern and the controls, while Figure 5.8 illustrates variations in the number of dental occlusal contacts between vertically classified groups.

Table 5.2: Clinical, dental and radiographic criteria of the subjects ***based on basic vertical facial classification.***

Group / Criteria	British norms*		Control n = 14		Long face n = 15	
Clinical						
Vertical (V)	-		Average		Increased	
Radiographic	Mean	SD	Mean	SD	Mean	SD
TAFH mm**	111.0	± 5.8	111.0	± 3.7	122.0	± 8.6
SN-MP°**	34.3	± 6.0	34.5	± 5.0	43.0	± 5.6
LAFH%	54.6	± 1.9	55.8	± 2.4	57.8	± 2.5
TPFH mm	71.4	± 4.3	69.0	± 3.0	70.0	± 7.6
RH mm	44.0	± 4.2	42.0	± 3.0	42.0	± 6.6
Dental						
Number of occlusal contacts**	-		26.0	± 5.0	14.0	± 3.0

*Female British norms (Bhatia and Leighton, 1993). **Significant p-value ≤ 0.05.

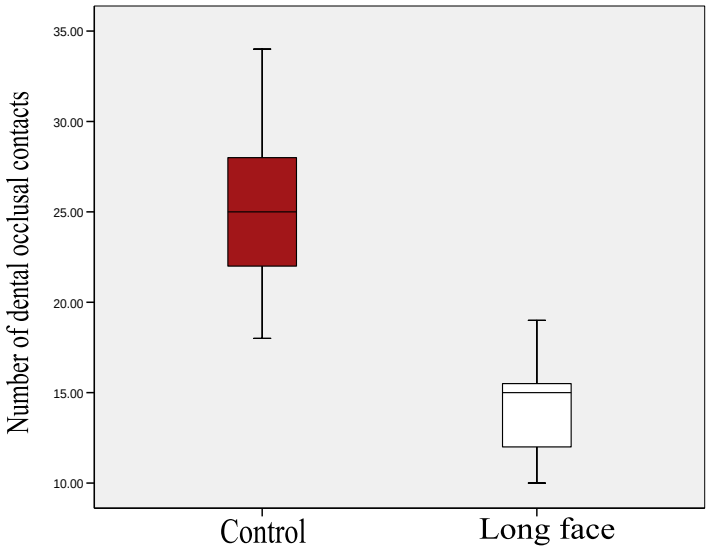


Figure 5.8: Variations in the number of dental occlusal contacts between the long face pattern and controls. *The number of dental occlusal contacts was significantly lower in the long face group compared to the controls. However, the long face group showed skewed data with a top shift of the median from the centre.*

5.5.3.2. Basic horizontal classification

The 29 subjects were classified based on their horizontal facial appearance regardless of vertical facial development. The classification generated three groups; 11 control, 5 Class II and 13 Class III patients. This classification was similar to the horizontal facial grouping that was used to analyse microarray data (Chapter 2, Section 2.7.8.1).

Table 5.3 summarises the clinical, radiographic and dental variations between the Class II, Class III and the controls, while Figure 5.9 illustrates the variation in the number of dental occlusal contacts between horizontally classified groups.

Table 5.3: Clinical, dental and radiographic criteria of the subjects ***based on basic horizontal facial classification.***

Group / **Criteria**	**British norms***		**Control n = 11**		**Class II n = 5**		**Class III n = 13**	
Clinical								
Horizontal (H)	-		Average		Retrognathic		Prognathic	
Radiographic	Mean	SD	Mean	SD	Mean	SD	Mean	SD
SNA°**	80.5	± 3.4	81.7	± 2.5	79.0	± 5.5	75.2	± 6.5
SNB°**	78.0	± 3.4	79.4	± 2.0	71.4	± 3.4	80.5	± 4.7
ANB°**	2.6	± 2.4	2.3	± 1.0	6.4	± 5.7	-5.3	± 3.6
Dental								
Number of occlusal contacts**	-		27.0	± 4.0	11.0	± 2.0	14.0	± 3.0

*Female British norms (Bhatia and Leighton, 1993). **Significant p-value ≤ 0.05.

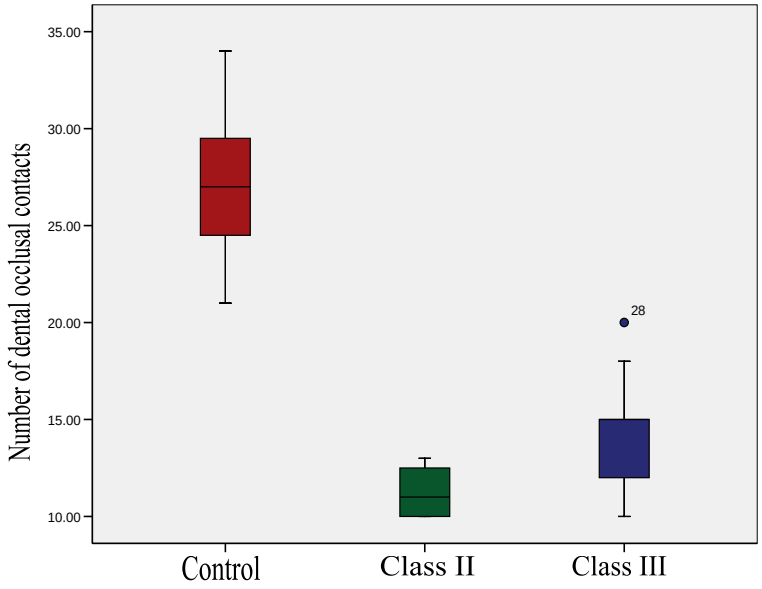


Figure 5.9: Variations in the number of dental occlusal contacts between Class II, Class III and controls. *The number of dental occlusal contacts was the lowest in the Class II followed by Class III patients compared to the controls. The Class II group showed skewed data, while the Class III group had one extreme case with increased number of dental occlusal contacts marked as an asterisk.*

5.5.3.3. Combined vertical and horizontal classification

The 29 subjects were classified based on both vertical and horizontal facial appearance. This classification generated four groups; 11 controls, 5 Class II long face, 10 Class III long face and 3 Class III average vertical facial patterns.

Table 5.4 summarises the clinical, radiographic and dental variations between the various combined vertical and horizontal groups, while Figure 5.10 illustrates the variation in the number of dental occlusal contacts between various combined patterns.

Table 5.4: Clinical, dental and radiographic criteria of the subjects ***based on combined vertical and horizontal facial patterns.***

Group / **Criteria**	**British norms***		**Control** n = 11		**Class II long face** n = 5		**Class III long face** n = 10		**Class III average face** n = 3	
Clinical										
V	-		Average		Increased		Increased		Average	
H	-		Average		Retrognathic		Prognathic		Prognathic	
H-variables	M	SD	M	SD	M	SD	M	SD	M	SD
SNA°**	80.5	± 3.4	81.7	± 2.5	79.0	± 5.5	73.6	± 6.0	80.6	± 5.2
SNB°**	78.0	± 3.4	79.4	± 2.0	72.6	± 2.3	80.0	± 4.7	83.6	± 4.0
ANB°**	2.6	± 2.4	2.3	± 1.0	6.4	± 5.7	-6.0	± 3.7	-3.0	± 2.0
V-variables										
LAFH%**	54.6	± 1.9	55.0	± 2.0	56.6	± 4.3	59.0	± 1.2	57.2	± 0.3
TAFH**	111.0	± 5.8	124.0	± 4.3	129.0	± 0.6	139.4	± 8.3	123.7	± 6.0
TPFH **	71.4	± 4.3	78.5	± 3.8	72.0	± 4.0	83.3	± 7.6	75.5	± 1.6
RH**	44.0	± 4.2	48.0	± 3.0	42.0	± 2.4	50.0	± 6.0	47.3	± 6.4
SN-MP°**	34.3	± 6.0	34.0	± 5.0	46.7	± 6.0	40.0	± 5.5	38.6	± 3.0
Dental										
Number of occlusal contacts**			27	± 4.0	11	± 2.0	13	± 2.0	18	± 2.0

*Female British norms (Bhatia and Leighton, 1993). **Significant p-value ≤ 0.05. V: Vertical. H: Horizontal. M: Mean. SD: Standard deviation.

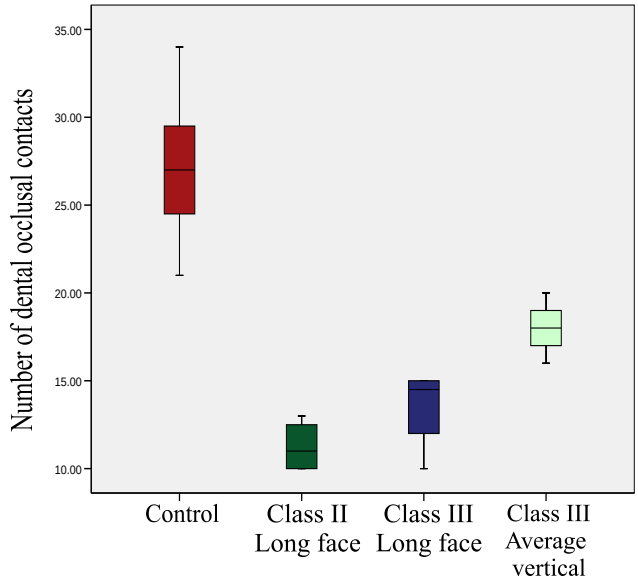


Figure 5.10: Variations in the number of dental occlusal contacts between Class II long faces, Class III long faces, Class III average vertical faces and controls. *The number of dental occlusal contacts was the lowest in the Class II long faces, followed by the Class III long faces and then the Class III average vertical faces compared to the controls. Both the controls and the Class III average vertical faces showed symmetric distribution of the data while both Class II and Class III long faces had skewed data.*

5.5.3.4. Subdivisions of the long face classification

The 29 subjects were classified based on their vertical facial appearance and the presence or absence of an anterior open bite (AOB), regardless of horizontal facial development. This classification generated three groups; 13 controls, 6 long faces with AOB and 9 long faces without AOB. The AOB in all patients of the long face with AOB group had an AOB higher than 3mm. Some of the long face without AOB patients had a minimal space (less than 1mm) between the upper and lower dentition which was counted as a dental AOB rather than skeletal AOB.

Table 5.5 summarises the clinical, radiographic and dental variations between the long face with AOB, long face without AOB and the controls, while Figure 5.11 illustrates the variations in the number of dental occlusal contacts between the various subdivisions of the long face pattern compared to the controls.

Table 5.5: Clinical, dental and radiographic criteria of the subjects ***based on long face subdivisions.***

Group **Criteria**	**British norms***		**Control** **n = 13**		**Long face with AOB** **n = 6**		**Long face without AOB** **n = 9**	
Clinical								
Vertical	-		Average		Increased		Prognathic	
Dental bite			Average		With AOB		Without AOB	
Radiographic	Mean	SD	Mean	SD	Mean	SD	Mean	SD
TAFH mm**	111.0	± 5.8	111.8	± 3.9	115.9	± 3.0	127	± 6.9
LAFH%**	54.6	± 1.9	55.7	± 2.4	56.5	± 2.6	58.8	± 2.2
TPFH mm **	71.4	± 4.3	70.0	± 3.2	62.8	± 4.7	76.0	± 6.9
RH mm**	44.0	± 4.2	42.8	± 3.3	37.2	± 4.4	45.5	± 5.7
SN-MP°**	34.3	± 6.0	34.6	± 4.8	47.2	± 5.4	39.8	± 5.4
Dental								
Number of occlusal contacts**	-		26.0	± 5.0	11.0	± 4.0	14.0	± 2.0
AOB mm			-	-	4.5	± 1.5	-	-

*Female British norms (Bhatia and Leighton, 1993). **Significant p-value ≤ 0.05. AOB: Anterior open bite.

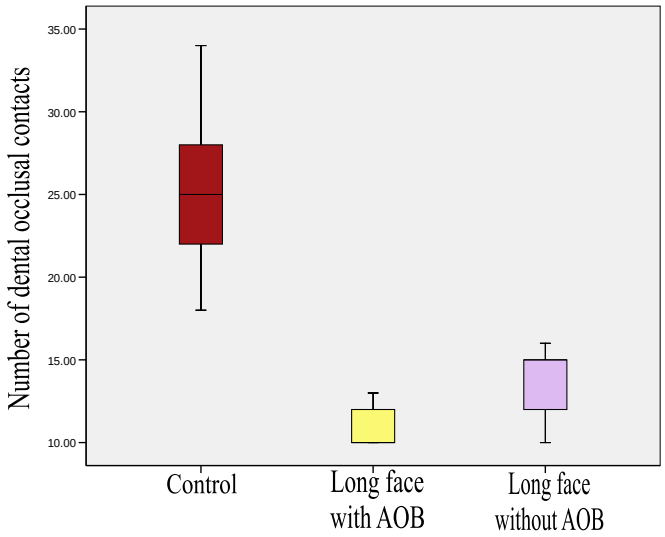


Figure 5.11: Variations in the number of dental occlusal contacts between long face with AOB, long face without AOB and controls. *The number of dental occlusal contacts was the lowest in the long face with AOB, followed by the long face without AOB when compared to the controls. Both long face subdivisions showed skewed data.*

5.6. DISCUSSION

Ideally, classification of patients for phenotype-genotype studies should be based on clearly defined craniofacial groupings, and should include clinical, dental and radiographic criteria. However, due to the ethical and technical issues associated with invasive types of human genetic studies as well as large individual variations, two main issues have been encountered: First, insufficient sample size to allow for comprehensive grouping and second, minimal numbers of controls with average vertical and horizontal craniofacial features. Other similar studies have encountered similar problems due to small sample sizes (Nelson-Moon et al., 1998; Gedrange et al., 2005; Suchak et al., 2009). Nevertheless, we report the largest number of control subjects (11) with average vertical and horizontal facial features.

Using the clinical, dental and radiographic criteria of the 29 subjects, it was possible to generate four sets of phenotypic classifications to assess the effect of various groupings on the masseter muscle qRT-PCR gene expression data analysis.

5.6.1. BASIC VERTICAL CLASSIFICATION

The problem with this classification was that some long face subjects exhibited horizontal discrepancies which were not taken into account. Furthermore, although the control group included patients with average vertical facial appearance, some of these patients had horizontal deformities. Such combinations in both the deformity and the control group may have an effect on the genotype-phenotype analysis.

5.6.2. BASIC HORIZONTAL CLASSIFICATION

The problem with this classification was that some of the Class III subjects had an average vertical facial appearance, while others had a long face pattern. Such combinations may also affect the genotype-phenotype analysis.

5.6.3. COMBINED VERTICAL AND HORIZONTAL CLASSIFICATION

Combining both vertical and horizontal criteria has addressed two main issues. First, from a vertical point of view, it has segregated between the Class II long face and the Class III long face patterns, which was the main problem with the basic vertical classification, as well as differentiating between the Class III long face and the Class III

average vertical face, which was the main problem of the basic horizontal classification. Second, from a horizontal point of view, this classification has addressed the issue of the skeletal differences between both subgroups of the Class III pattern, where the majority of the Class III long face patients showed a retruded maxilla and a normal mandible subtype, while all Class III average vertical faces had a normal maxilla and a prognathic mandible subtype. However, an obvious disadvantage was that the Class III average face group had a reduced sample size (3 patients).

5.6.4. SUBDIVISIONS OF THE LONG FACE

Combining patients with similar vertical skeletal and dental criteria had the advantage of detecting differences between the various subdivisions of the long face pattern. These were evident clinically and radiographically between the long face with AOB (reduced TPFH, RH and increased SN-MP) and the long face without AOB (increased TPFH, RH and normal SN-MP). However, the disadvantage was that the horizontal pattern was not addressed since Class III average patients were considered part of the controls.

5.7. SUMMARY AND CONCLUSIONS

Each craniofacial classification has its own advantages and disadvantages. However, depending on the aims of the project, the sample size and the craniofacial criteria of the recruited subjects, one can use various classifications to answer the proposed research question. Using the various clinical, dental and radiographic features of the subjects it was possible to generate four sets of phenotypic data to be further analysed in relation to the masseter muscle genotype.

Chapter 6. Masseter muscle genotype in relation to various craniofacial phenotypes: Analysis of quantitative RT-PCR data

6.1. INTRODUCTION

Investigation of myosin heavy chain (*MYH*) gene expressions in previous masseter muscle genotype-phenotype research has been based on either vertical (Nelson-Moon et al., 1998; Suchak et al., 2009) or horizontal (Gedrange et al., 2005 and 2006; Maricic et al., 2008) craniofacial deformities. However, no previous study has assessed both vertical and horizontal facial parameters of the subjects within the same study, and its effect on masseter muscle gene expression compared to patients with average vertical and horizontal facial features.

Also, the current data set exhibited varying degrees of maxillary, mandibular and dental discrepancies as shown by radiographic variables and the number of dental occlusal contacts (all subjects's criteria are presented in Chapter 5, Section 5.5.2). The introduction of correlation statistical analyses into genotype-phenotype studies has proved useful in such situations (Sabaghnia et al., 2006). This type of analysis dictates the relationship between one dependent variable (gene expression) and another independent variable (e.g. varying degrees of the number of dental occlusal contacts).

Using the genetic data generated from the microarray experiment (Chapter 4 -five novel genes) and the phenotypic data generated from the previous chapter (Chapter 5 -four phenotypic classifications), the aims of the current analyses were to:

- Assess masseter muscle gene expression of the novel genes ascertained from the microarray experiment (KIAA1671, DGCR6, NDRG2, SERGEF and LOC730245) and the previously investigated myosin heavy chain genes (*MYH*1, 2, 3, 6, 7 and 8) in relation to basic vertical, basic horizontal, long face subdivisions and combined vertical and horizontal phenotypic classifications of the subjects.

- Undertake a correlation analysis to assess the effect of various vertical and horizontal cephalometric parameters on masseter muscle gene expression of novel and *MYH* genes.

- Undertake a correlation analysis to assess the effect of the number of dental occlusal contacts on masseter muscle gene expression of novel and *MYH* genes.

6.2. MATERIALS AND METHODS

6.2.1. PHENOTYPIC IDENTIFICATION

The 29 subjects were classified four times based on: 1) Basic vertical; 2) Basic horizontal; 3) Combination of vertical and horizontal craniofacial parameters; 4) Long face subdivisions (details of each classification are presented in Chapter 5, Section 5.5.3).

6.2.2. GENOTYPIC IDENTIFICATION

6.2.2.1. RNA samples

A total of 29 RNA samples (details of RNA quality control for each sample is presented in Chapter 3 Figure 3.7) were used to assess the gene expression of novel (KIAA, DGCR6, NDRG2, SERGEF and LOC) and previously tested genes (*MYH* 1, 2, 3, 6, 7 and 8) of the masseter muscle in relation to different clinical and cephalometric craniofacial features as well as dental occlusal contacts.

6.2.2.2. TaqMan® assays selected

Each gene of interest, including the reference gene (GAPDH), was tested in quadruplicate reactions (details of the laboratory protocol are available at Appendix C). A total of 12 TaqMan® gene expression assays were therefore undertaken. Eleven of the 12 assays were ready to use, tested by the company (Applied Biosystems) and guaranteed with 92-100% amplification efficiency. Only one gene assay, representing one of the novel genes ascertained from the microarray experiment (Hypothetical protein LOC730245 gene) had not been previously manufactured. Special software downloaded from the Applied Biosystems website called file builder® v3.1.0 was used by the investigator to conduct bioinformatics on the sequence of interest (derived from the NetAffyx target sequence information of the U133 Plus 2.0 array GeneChip®). The gene sequence was then submitted to the company for the construction of a TaqMan® gene expression assay (details of the TaqMan® gene expression assay ID numbers and

GenBank mRNA sequence reference, of the genes of interest, are available at Appendix B).

Following delivery of the customised assay, an efficiency test was conducted by the investigator to ensure 92-100% amplification efficiency of the newly customised assay. The test was performed using a series of 10-fold dilutions of one cDNA sample as a template and was conducted 5 times for both the customised assay and the GAPDH assay (as a control). The settings of the real-time PCR plate and machine were conducted as described in the previous section. The Ct values were exported from the qRT-PCR machine and were plotted against the log transformation of the concentration. The efficiency % (Ex) was calculated using the equation: $Ex = 10^{(-1/slope)} -1 \times 100$. The slope (y) was derived from the graph. The graph was generated using Excel® software 2003. Once the graph was generated, the slope was automatically calculated by the software (Figure 6.1).

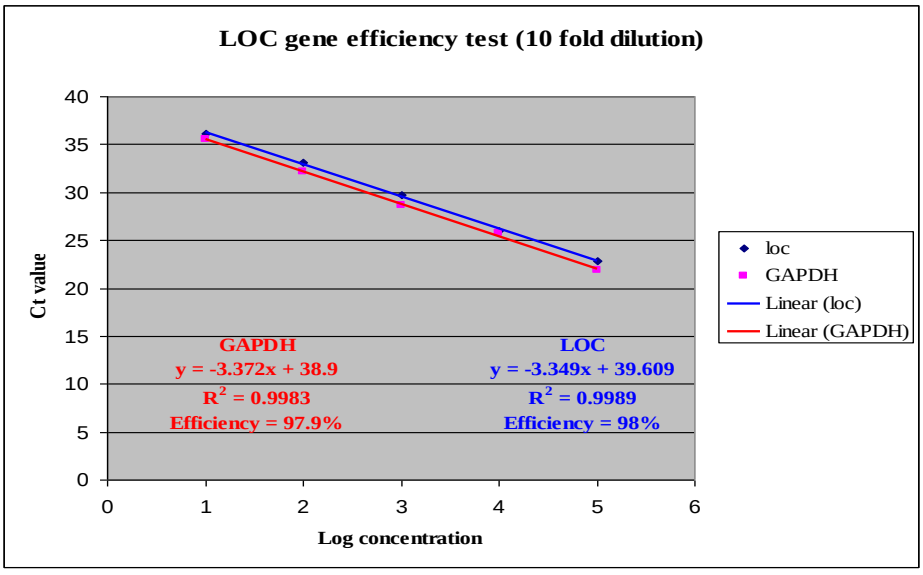


Figure 6.1: LOC gene efficiency test. *The Ct values presented on the y-axis indicate the cycle at which the gene expression was first detected, plotted against the log transformation of the total RNA concentration on the x-axis. In order to use the* $2^{-\Delta\Delta Ct}$ *equation to calculate the gene expression intensity, each gene has to have a constant correlation between the Ct value and the total RNA concentration (i.e. the higher the concentration the lower the Ct value – the earlier the gene is detected on the real-time PCR machine). The graph shows a strong constant regression correlation demonstrated by the straight line and a correlation coefficient of* R^2*= 0.99 between the Ct value and the sample concentration where the higher the concentration, the earlier the Ct cycle of the LOC gene. The GAPDH gene was also tested using the same sample to act as a control. y, slope;* R^2*, correlation regression coefficient, the closer this value to 1 the stronger the correlation.*

6.2.2.3. Normalisation and generation of gene intensity values

Using the equation $2^{-\Delta\Delta Ct}$, 11 gene expression values were generated for each individual representing the five novel and the six *MYH* genes (details of normalisation and calculation of gene intensity values are presented in Chapter 2, Section 2.7.5.5).

6.2.3. GENOTYPE-PHENOTYPE ANALYSIS

6.2.3.1. Masseter muscle gene expression in relation to basic vertical phenotypes (long face vs. control)

Variation in the masseter muscle gene expression of novel and previously determined genes between the control and long face patients was assessed using the Mann Whiney U-test (SPSS v14 -non-parametric type of analysis). A p-value of ≤ 0.05 was considered to be statistically significant.

6.2.3.2. Masseter muscle gene expression in relation to basic horizontal phenotypes (Class II vs. Class III vs. control)

Variation in the masseter muscle gene expression of novel and previously determined genes between the control, Class II and Class III patients was assessed using the Kruskal Wallis test (SPSS v14 -non-parametric type of analysis). A p-value of ≤ 0.05 was considered to be statistically significant.

6.2.3.3. Masseter muscle gene expression in relation to combined vertical and horizontal phenotypes (Class II long face vs. Class III long face vs. Class III average vertical face vs. control)

Variation in the masseter muscle gene expression of novel and previously determined genes between the control, Class II long face, Class III long face and Class III average vertical face patients was assessed using the Kruskal Wallis test (SPSS v14). A p-value of ≤ 0.05 was considered to be statistically significant.

6.2.3.4. Masseter muscle gene expression in relation to long face subdivisions (long face with AOB vs. long face without AOB vs. control)

Variation in the masseter muscle gene expression of novel and previously determined genes between the control, long face with AOB and long face without AOB was

assessed using the Kruskal Wallis test (SPSS v14). A p-value of ≤ 0.05 was considered to be statistically significant.

6.2.3.5. Correlation between masseter muscle gene expression and craniofacial cephalometric variables

The Spearman's correlation coefficient analysis (SPSS v14 -non-parametric correlation) was used to establish the relationship between various cephalometric variables (vertical -LAFH%, TAFH, TPFH, RH and SN-MP angle and horizontal -SNA, SNB and ANB angles) and masseter muscle gene expression. A p-value of ≤ 0.05 was considered to be statistically significant.

6.2.3.6. Correlation between masseter muscle gene expression and the number of dental occlusal contacts

The Spearman's correlation coefficient analysis (SPSS v14 -non-parametric correlation) was used to establish the relationship between the number of dental occlusal contacts and masseter muscle gene expression. A p-value of ≤ 0.05 was considered to be statistically significant.

6.3. RESULTS

6.3.1. LONG FACE vs. CONTROLS

None of the novel or the *MYH* genes was found to be statistically significantly different between long face patients and the controls when only vertical facial criteria were considered.

6.3.2. CLASS II vs. CLASS III vs. CONTROLS

The *MYH*3, *MYH*6, *MYH*7, SERGEF and NDRG2 gene expressions were all found to be significantly different between various horizontal groups. The *MYH*3, *MYH*6 and *MYH*7 were all up-regulated in Class II patients compared to both Class III and the controls (p-values 0.049, 0.042 and 0.011, respectively). Both the SERGEF and NDRG2 genes were down-regulated in the Class III group compared to the Class II pattern and the controls with p-values of 0.016 and 0.044, respectively. Figure 6.2 is a

graphical representation of gene expression variations between Class II, Class III and the controls.

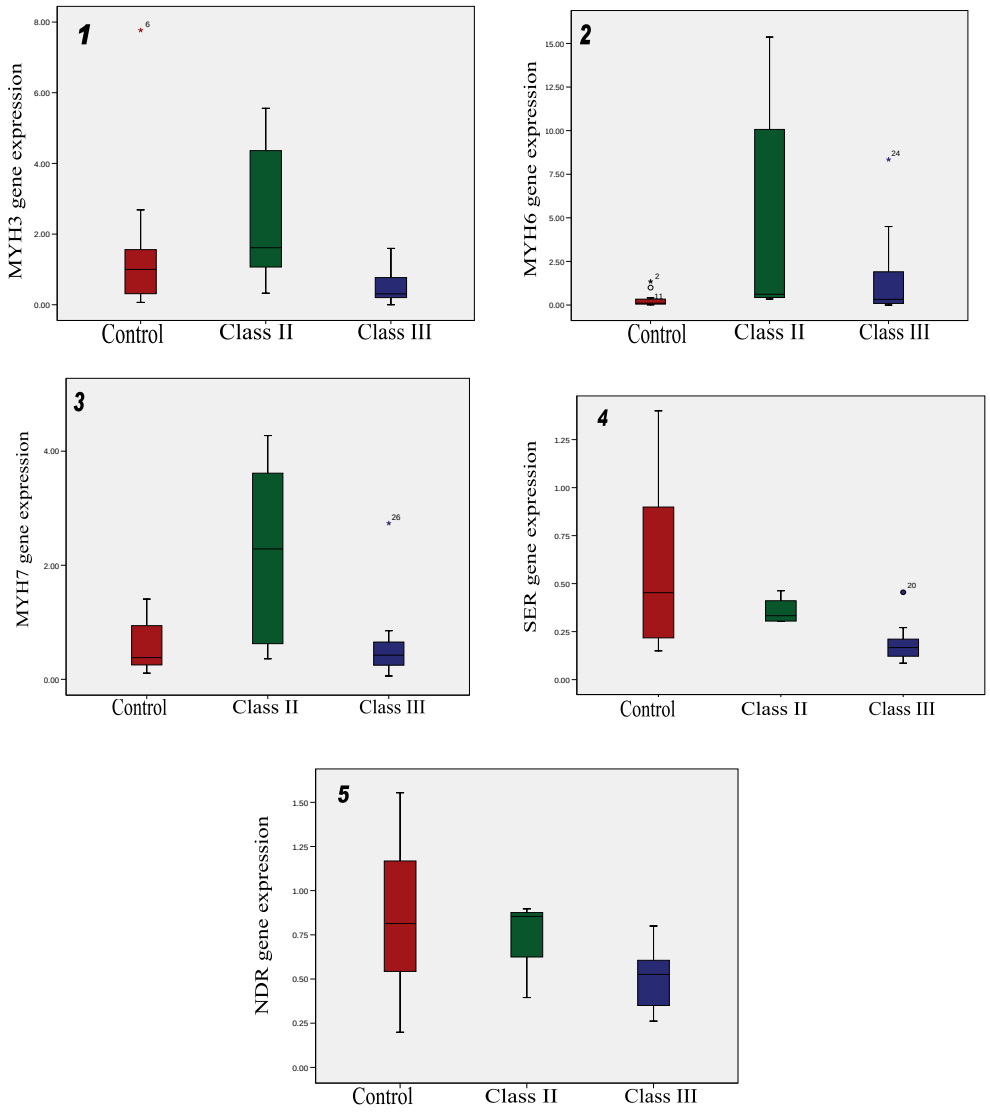


Figure 6.2: Masseter muscle gene expression variations between Class II, Class III and controls. *Generally, large individual variations and skewed data were noted in all three groups.* ***1)*** *The MYH3 gene expression was up-regulated in the Class II group compared to both Class III and the controls.* ***2)*** *The MYH6 was up-regulated in the Class II group compared to both Class III and the controls. Two extreme cases were found in the control and the Class III group and were denoted as an asterisk.* ***3)*** *The MYH7 was up-regulated in the Class II pattern compared to Class III patients and the controls. The Class III pattern had one extreme case denoted as an asterisk.* ***4)*** *The SERGEF gene was down-regulated in the Class III group compared to Class II and the controls. The Class III group had one extreme case marked as an asterisk.* ***5)*** *The NDRG2 gene was down-regulated in the Class III group compared to Class II and the controls.*

6.3.3. CLASS II LONG FACE vs. CLASS III LONG FACE vs. CLASS III AVERAGE VERTICAL FACE vs. CONTROLS

The *MYH*7, SERGEF, NDRG2 and KIAA genes were found to be significantly different when both vertical and horizontal craniofacial features were taken into account. The *MYH*7 gene expression was up-regulated in Class II long face patients compared to both Class III subgroups and the controls (p-values 0.023). Both NDRG2 and KIAA genes were up-regulated in Class II long faces and down-regulated in both Class III subgroups (long and average vertical faces) compared to the controls (p-values 0.029 and 0.021, respectively). The SERGEF gene, on the other hand, was down-regulated in both the Class III long faces and Class III average vertical faces compared to both Class II long face patients and the controls (p-value 0.004). Figure 6.3 is a graphical representation of gene expression variations between Class II long faces, Class III long faces, Class III average vertical faces and the controls.

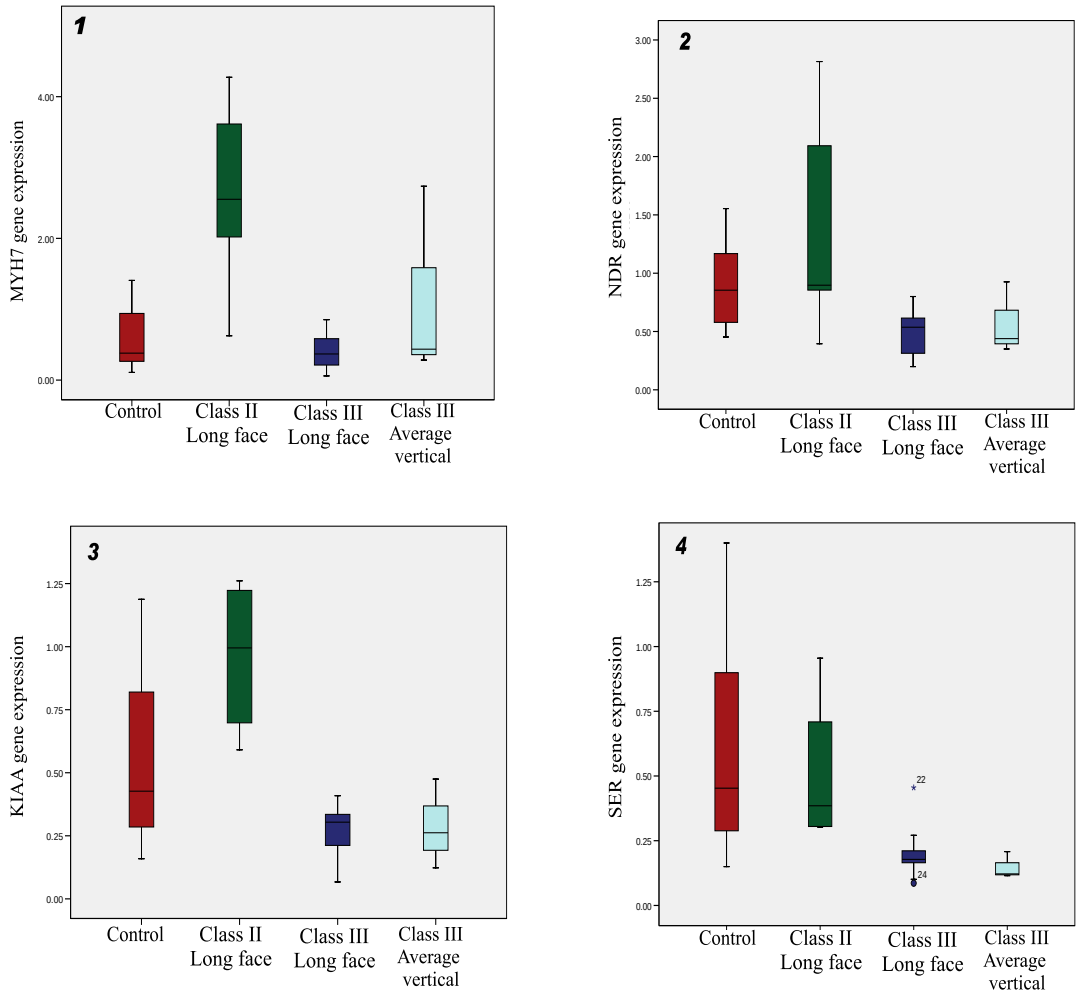


Figure 6.3: Masseter muscle gene expression variations between Class II long faces, Class III long faces, Class III average vertical faces and controls. 1) *The MYH7 gene expression was up-regulated in the Class II long face group compared to both Class III subgroups and the controls. Large individual variations were noted in the Class II long face and Class III average vertical face groups.* ***2)*** *The NDRG2 gene expression was up-regulated in Class II long face group and down-regulated in both Class III subgroups compared to the controls. However, large individual variations were evident in Class II long face individuals.* ***3)*** *The KIAA gene expression was up-regulated in Class II long face patients and down-regulated in both Class III subgroups compared to the controls. Both the controls and the Class II long face groups had large individual variations, while both Class III subgroups showed more coherent data.* ***4)*** *The SERGEF gene was down-regulated in the both Class III subgroups compared to both Class II long face and the controls. The Class III long face pattern had one outlier sample marked as a circle and one extreme value marked as an asterisk, while the Class III average vertical face had largely skewed data.*

6.3.4. LONG FACE WITH AOB vs. LONG FACE WITHOUT AOB vs. CONTROLS

Both the *MYH7* and KIAA1671 genes were up-regulated in long face individuals with AOB compared to long face patients without AOB and the controls (p-values 0.008 and 0.005, respectively). Figure 6.4 is a graphical representation of gene expression variations between long face with AOB, long face without AOB and the controls.

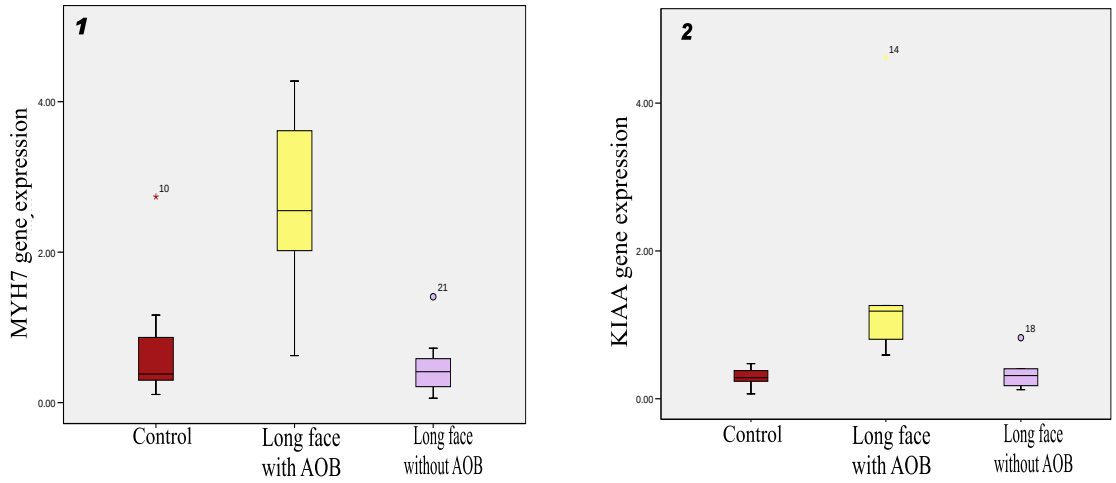


Figure 6.4: Masseter muscle gene expression variations between long faces with AOB, long faces without AOB and controls. 1) *The MYH7 gene expression was up-regulated in the long face with AOB group compared to both long face without AOB and the controls. Large individual variations were evident between the long face with AOB patients while the long face without AOB had one outlier sample marked as a circle. The control group had skewed data with one extreme value marked as an asterisk.* ***2)*** *The KIAA gene expression was up-regulated in long face with AOB compared to both long face without AOB and the controls. Both subdivisions of the long face had skewed data.*

6.3.5. CORRELATION BETWEEN MASSETER MUSCLE AND GENE EXPRESSION

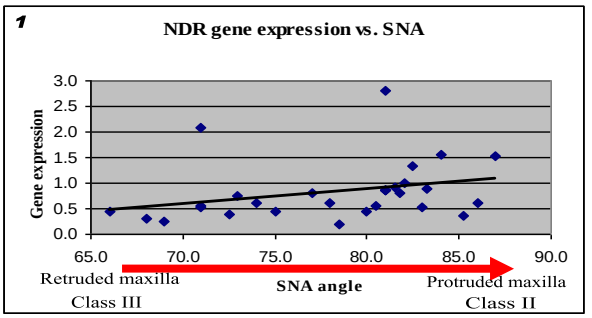
- Vertical variables

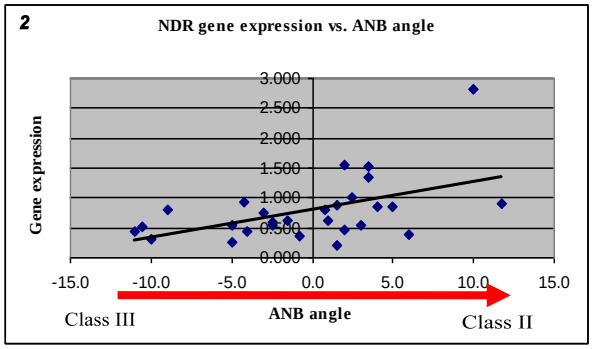
No correlation was found between the gene expression of the novel or the *MYH* genes and any of the vertical cephalometric variables (TAFH, LAFH%, TPFH, RH and SN-MP).

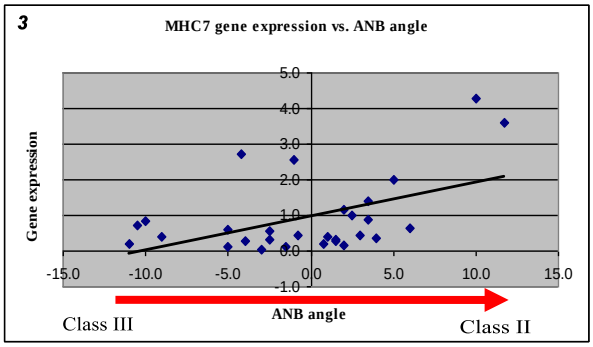
- Horizontal variables

The NDRG2 gene expression was positively related to the SNA angle. The higher the SNA angle as in Class II patients, the greater the gene expression (p-value of 0.006). Furthermore, the *MYH*7, SERGEF and NDRG2 genes were positively related to the ANB angle (p-values 0.029, 0.006, 0.012, respectively). The higher the ANB angle, as in Class II patients, the greater the *MYH*7 gene expression (encode slow contracting protein isoform). The lower the ANB angle, as in Class III individuals, the lower the SERGEF gene expression. The higher the ANB angle, as in Class II patients, the greater the NDRG2 gene expression. Figure 6.5 is a graphical representation of the correlation between horizontal cephalometric variables and the masseter muscle gene expression.

Masseter muscle genotype
vs.
Radiographic phenotype







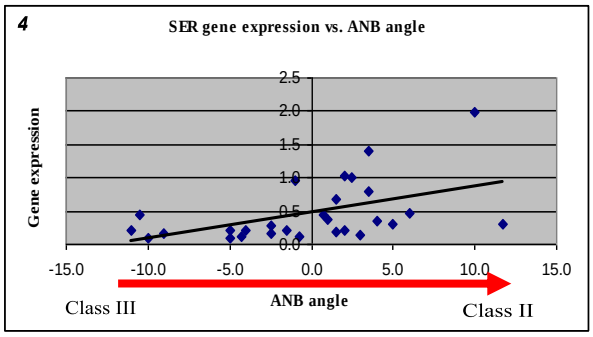


Figure 6.5: Masseter muscle gene expression correlation to cephalometric variables. *Generally, the masseter muscle gene expression showed a positive correlation to the SNA and the ANB angles of the horizontal cephalometric variables* ***1)*** *The higher the SNA°, as in Class II phenotypes (protruded maxilla), the greater the NDRG2 gene expression.* ***2)*** *The higher the ANB°, as in Class II phenotypes (distal maxillary-mandibular horizontal relationship), the greater the NDRG2 gene expression.* ***3)*** *The greater the ANB°, as in Class II patients, the greater the MYH7 gene expression.* ***4)*** *The lower the ANB°, as in Class III phenotypes (negative maxillary mandibular horizontal relationship), the lower the SERGEF gene expression.*

6.3.6. CORRELATION BETWEEN MASSETER MUSCLE GENE EXPRESSION AND THE NUMBER OF DENTAL OCCLUSAL CONTACTS

Both SERGEF and NDRG2 gene expressions were significantly positively related to the number of dental occlusal contacts (p-values 0.019 and 0.032, respectively). The lower the number of dental occlusal contacts, as in Class II, Class III and long face patients, the lower the SERGEF and NDRG2 gene expressions. Figure 6.6 is a graphical representation of the correlation between the number of dental occlusal contacts and the masseter muscle gene expression.

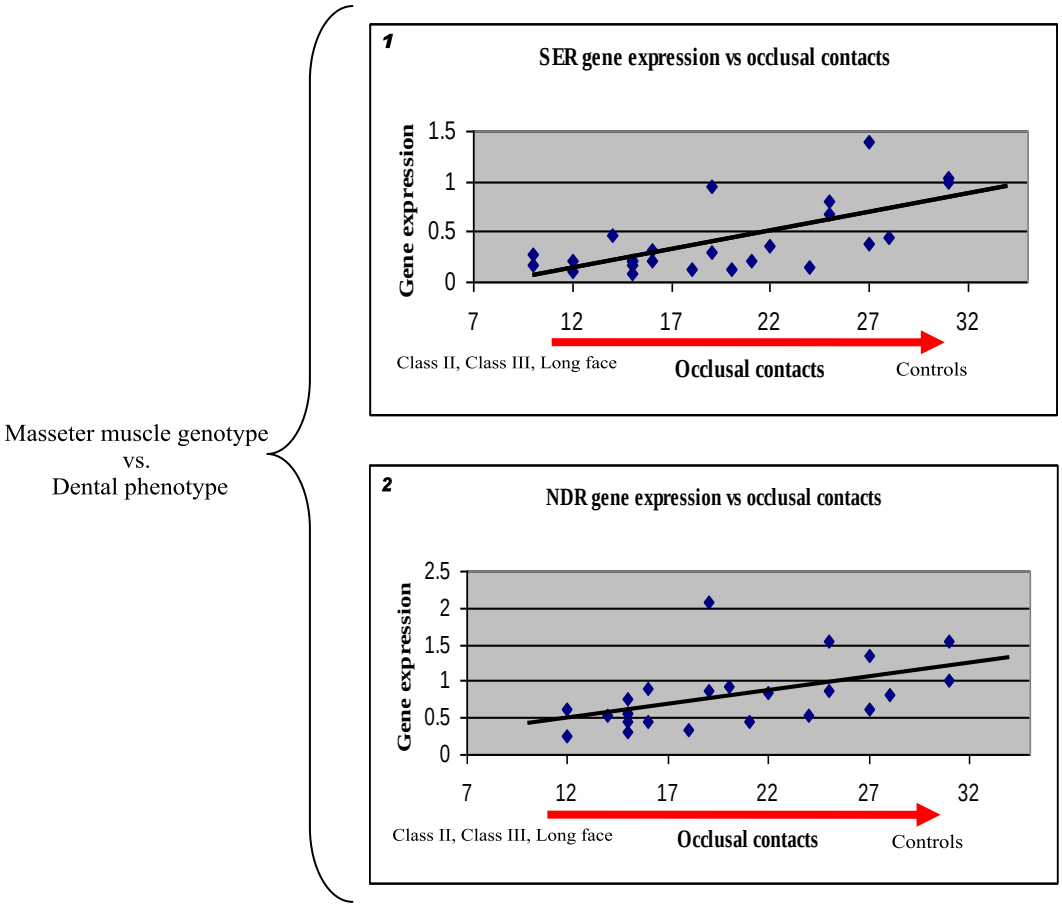


Figure 6.6: masseter muscle gene expression correlation to the number of dental occlusal contacts. *Generally, a positive correlation was observed between the masseter muscle gene expression and the number of dental occlusal contacts.* ***1 and 2)*** *The lower the number of dental occlusal contacts, as in Class II, Class III and long face patients (with and without AOB), the lower the gene expression of both the SERGEF and NDRG2 genes.*

6.4. DISCUSSION

In the current chapter five main issues have been addressed. First, the effect of various phenotypic classifications on the genotypic analysis. Second, variations in masseter muscle gene expression in relation to different craniofacial phenotypes. Third, the effect of various cephalometric variables on masseter muscle gene expression. Fourth, the effect of the number of dental occlusal contacts on the masseter muscle gene expression. Fifth, validation of microarray data.

6.4.1. THE EFFECT OF VARIOUS PHENOTYPIC CLASSIFICATIONS ON GENOTYPIC ANALYSIS

It has been demonstrated that the genotypic analysis can be affected by the phenotypic classification used, and the need to segregate both Class II and Class III patterns is mandatory during vertical classification even though they may have similar vertical facial appearance. Gedrange and colleagues (2006) in a study of 10 patients with horizontal deformities (5 Class II and 5 Class III subjects) found that the expression of *MYH*2 and *MYH*7 was higher in Class II patients than in Class III individuals. Combining both Class II long face and Class III long face patterns as one single group may camouflage the true effect of the vertical deformity. This was evident in the present study. When the patients were classified based solely on vertical facial parameters into 15 long face patients (including 5 Class II and 10 Class III patients) and 14 average vertical controls (including 11 average vertical and horizontal subjects and 3 Class III average vertical face patients), none of the novel or the *MYH* genes were found to behave significantly differently. However, when the same patients were regrouped based on both vertical and horizontal facial parameters into 5 Class II long faces, 10 Class III long faces, 3 Class III average faces and 11 controls (average vertical and horizontal), the *MYH*7, NDRG2, KIAA1671 and SERGEF genes were all found to be differentially expressed between the various classified groups.

Furthermore, when considering subdivisions of the long face pattern using vertical classifications, this also had an impact on masseter muscle gene expression analysis. This was clearly shown when none of masseter muscle genes were differentially expressed when the patients were classified into only long face and average controls. However, *MYH*7 and KIAA1671 genes were found to be up-regulated in relation to

long faces with AOB compared to long faces without AOB and the controls when the patients were regrouped based on the various subdivisions of the long face pattern. This may suggest that different gene expression mechanisms are related to the long face pattern with and without AOB. However, cautious interpretation of the data is required as the majority of the long faces with AOB were Class II patients, while all the long faces without AOB individuals exhibited a Class III pattern (clinical and dental details of subjects are presented in Chapter 5, Figure 5.7) which may have masked the vertical effect. Further investigation on a larger sample size with less variability and more coherent data is recommended.

6.4.2. MASSETER MUSCLE GENE EXPRESSION VARIATIONS BETWEEN VARIOUS CRANIOFACIAL DEFORMITIES

Few studies have investigated the different *MYH* gene expression isoforms in relation to different craniofacial patterns (Maricic et al., 2008; Suchak et al., 2009). As already stated, these studies have been based on either vertical (Nelson-Moon et al., 1998; Suchak et al., 2009) or horizontal (Gedrange et al., 2006; Maricic et al., 2008) facial appearance and none have taken into account both vertical and horizontal criteria of the subjects. Furthermore, the studies have included few or no control patients with average vertical and horizontal craniofacial parameters for comparison (Suchak et al., 2009). The current research has investigated all six *MYH* genes (*MYH*1, *MYH*2, *MYH*3, *MYH*6, *MYH*7 and *MYH*8) as well as the novel genes ascertained from the current microarray data in relation to vertical, horizontal, combined vertical and horizontal criteria and the subdivisions of the long face pattern.

- Long face vs. controls: None of the novel or *MYH* masseter muscle genes were found to be significantly different when the patients were classified based on only vertical facial parameters including 15 long faces and 14 average controls. These findings were similar to the results of Suchak and colleagues, (2009) who investigated all six *MYH* genes in 9 long face patients compared to 1 average control. Furthermore, the qRT-PCR gene expression analysis of the novel genes did not support the microarray results, where both the KIAA1671 and DGCR6 genes were found to be down-regulated in long face patients compared to the controls.

- Class II vs. Class III vs. controls: The embryonic *MYH*3, slow *MYH*6 and slow *MYH*7 genes were all found to be up-regulated in 5 Class II patients compared to 13 Class III and 11 control patients. These results concur with the findings of Gedrange and colleagues, (2006) in a study of 5 Class II and 5 Class III patients who found that *MYH*7 gene expression was higher in Class II patients compared to Class III individuals (Gedrange et al., 2006). However, their study did not include the *MYH*3 or *MYH*6 genes. As for the novel genes, SERGEF and NDRG2 were down-regulated in Class III patterns compared to both the Class II and the controls. qRT-PCR analysis of SERGEF gene expression confirmed the microarray results. However, this did not support the other microarray finding where the NDRG2 was down-regulated in Class II patients compared to Class III individuals.

- Class II long faces vs. Class III long faces vs. Class III average vertical faces vs. controls: Instead of using basic vertical or simple horizontal grouping, an adaptation of the classification recommended by Sassouni, (1969), which takes into account both vertical and horizontal patterns, was used. The *MYH*7, NDRG2 and KIAA1671 were found to be up-regulated in 5 Class II long face patients compared to both Class III subgroups (10 long and 3 average vertical faces) and 11 controls (average vertical and horizontal). Interestingly, the SERGEF gene expression was not affected by splitting the Class III group into long and average vertical faces and was down-regulated in relation to all Class III subgroups. This suggests that the SERGRF gene expression may be affected more by horizontal patterns than vertical facial development. This classification has not been used previously by other investigators.

- Long faces with AOB vs. long faces without AOB vs. controls: The *MYH*7 and KIAA1671 were up-regulated in relation to 6 long faces with AOB compared to 9 long faces without AOB and 14 controls. As explained earlier, the majority of the long faces with AOB were Class II patients while all long faces without AOB individuals exhibited a Class III pattern and therefore, careful interpretation of this data is recommended. This classification has not been

previously used in masseter muscle genotype-phenotype studies and a larger sample size should be investigated in future.

6.4.3. MASSETER MUSCLE GENE EXPRESSION vs. CEPHALOMETRIC VARIABLES

The current data indicate that none of the vertical cephalometric variables were found to be correlated to masseter muscle gene expression. This finding concurs with one of the results of Suchak and colleagues, (2009), who found no correlation between the expression of any of the six *MYH* genes and the LAFH%.

Nevertheless, the present data indicate that the masseter muscle gene expression of the NDRG2 gene was positively correlated to the SNA and ANB angles, while the *MYH7* and SERGEF were positively correlated only to the ANB angle. The higher the SNA and ANB angles, as in the Class II pattern compared to the controls, the greater the NDRG2 gene expression. The higher the ANB angle, as in Class II patients, the greater the *MYH*7 gene expression. The lower the ANB angle, as in Class III patients, the lower the SERGEF gene expression.

6.4.4. MASSETER MUSCLE GENE EXPRESSION vs. THE NUMBER OF DENTAL OCCLUSAL CONTACTS

The masseter muscle gene expressions of only SERGEF and NDRG2 genes were positively correlated to the number of dental occlusal contacts. The lower the number of occlusal contacts, as in the long faces, Class II and Class III patients, the lower the SERGEF and the NDRG2 gene expression. None of the *MYH* genes or the remaining three novel genes have been found to be correlated to the number of dental occlusal contacts.

Nelson-Moon and co-workers, (1998), investigated the correlation between *MYH*1, *MYH*2, *MYH*3, *MYH*6, *MYH*7 and *MYH*8 and the number of dental occlusal contacts in a group of patients with vertical facial discrepancies. An inverse correlation was found between the embryonic *MYH*3 gene expression and the number of dental occlusal contacts (i.e. the lower the number of occlusal contacts, the higher the *MYH*3 gene expression). Although the current data does not directly support their results, the

correlation analysis showed that the Class II patients had the lowest number of occlusal contacts and the highest *MYH*3 gene expression compared to Class III individuals and the controls. The reason for the variations between the studies could have been attributed to the fact that the sample used in Nelson-Moon and colleagues' study, (1998) included 9 long face patients and 6 average controls, all of whom had pre-surgical orthodontics prior to orthognathic surgery. This indicates that the controls were patients with horizontal deformities but had average vertical facial features. The present data included a broad range of deformities compared to patients with average vertical and horizontal features.

6.5. SUMMARY AND CONCLUSIONS

- Both vertical and horizontal facial patterns should be taken into account when considering phenotype-genotype analysis.

- The qRT-PCR data suggests that the *MYH*3 (encode embryonic MyHC protein), *MYH*6 (encode α-cardica slow-contracting MyHC protein) and *MYH*7 (encode slower-contracting MyHC protein) genes were all up-regulated in Class II individuals compared to Class III and control subjects.

- Both SERGEF and NDRG2 gene expression was down-regulated in Class III subjects compared to the controls.

- The masseter muscle expression of the NDRG2 gene was positively correlated to both SNA and ANB angles, while the *MYH*7 and SERGEF gene expressions were positively correlated to the ANB angle.

- The masseter muscle expression of NDRG2 and SERGEF genes were correlated to the number of dental occlusal contacts.

- The SERGEF gene showed consistent expression results when tested by both microarray and qRT-PCR techniques.

Chapter 7. General discussion

7.1. SUMMARY OF THE RESULTS

The current genotype-phenotype study was designed based on three main levels: firstly, to identify masseter muscle genotypes; secondly, to identify craniofacial phenotypes; thirdly, to relate masseter muscle genotypes to craniofacial phenotypes according to clinical, dental and radiographic features. A summary of the main results for the current research is presented in Table 7.1.

Table 7.1: Summary of the main results for the current research.

The null hypothesis	Techniques implemented	Result
1) No novel masseter muscle genes can be identified in relation to craniofacial patterns, when using the microarray technology confirmed by quantitative RT-PCR (qRT-PCR).	-Microarrays -qRT-PCR	-Null hypothesis rejected -SERGEF gene was down-regulated in Class III pattern using both microarray and qRT-PCR.
2) There is no relationship between masseter muscle gene expression and various combinations of vertical and horizontal craniofacial patterns.	-qRT-PCR	-Null hypothesis rejected. - *MYH*3, *MYH*6 and *MYH*7were up-regulated in Class II patients. - SERGEF and NDRG2 down-regulated in Class III individuals.
3) There is no correlation between masseter muscle gene expression and various vertical and horizontal cephalometric variables.	-qRT-PCR data vs. cephalometric variables.	-Null hypothesis rejected. - NDRG2 positively correlated to SNA and ANB angles. - *MYH*7 and SERGEF positively correlated to the ANB angle.
4) There is no correlation between masseter muscle gene expression and the number of dental occlusal contacts associated with craniofacial deformities.	-qRT-PCR data vs. dental occlusal contacts.	-Null hypothesis rejected. - NDRG2 and SERGEF positively correlated to the number of dental occlusal contacts.

7.2. IDENTIFICATION OF MASSETER MUSCLE GENOTYPE

This study is the first to report the use of microarray technology in relation to the masseter muscle gene expression of non-syndromic patients with variable vertical and horizontal craniofacial deformities. Using this technology, a candidate gene list has been identified for patients with long face features (12 up- and 19 down-regulated genes compared to the controls), Class II (3 up- and 9 down-regulated genes compared to the controls) and Class III patterns (15 up- and 21 down-regulated genes compared to the controls) that were not reported previously in relation to the masseter muscle (Chapter 4, Tables 4.3 and 4.4). Any of these genes would warrant further investigation in relation to craniofacial phenotypes.

Out of the candidate gene list, 5 "novel" genes were identified based on statistical analysis, and these warrant further investigation alongside the "informative" *MYH* genes in relation to craniofacial phenotypes. The microarray gene expression status of one (SERGEF), out of the five novel genes, showed consistent results between both microarray and qRT-PCR data, while the remaining four genes (DGCR6, KIAA1671, LOC730245 and NDRG2) presented inconsistent results. However, this does not rule out their involvement in relation to craniofacial deformities, and further investigation on genomic and proteomic levels is recommended. Table 7.2 summarises the microarray and the qRT-PCR gene expression status of the 5 novel genes.

Table 7.2: Gene expression status of the five novel genes.

Gene name	Gene symbol	Microarray data*	qRT-PCR data**
Secretion Regulating Guanine nucleotide Exchange Factor	SERGEF	*Down-R* in Class III compared to Ctrl	*Down-R* in Class III compared to Ctrl
KIAA1671	KIAA1671	*Down-R* in LF compared to Ctrl	*Up-R* in LF AOB compared to LF no AOB and Ctrl
N-myc Downstream Regulated Gene family member 2	NDRG2	*Down-R* in Class II compared to Class III	*Up-R* in Class II compared to Class III
DiGeorge syndrome Critical Region gene family member 6	DGCR6	*Down-R* in LF compared to Ctrl	No significant results p-value 0.232
Hypothetical protein Locus 730245	LOC730245	*Up-R* in Class III compared to Ctrl	No significant results p-value 0.361

*Significant p-value ≤ 0.001. **Significant p-value ≤ 0.05. R: Regulated. Ctrl: Control. LF: Long face. AOB: Anterior open bite.

Both microarray (Rogojina et al., 2003) and qRT-PCR (Levesque-Sergerie et al., 2007) are sensitive gene expression techniques. A possible explanation for the variations between both techniques may be to the nature of the microarray experiment where thousands of genes are tested and false positive results are more likely (Shi et al., 2006). Both technologies have been reported with different technical and analytical procedures (Table 7.3). However, one can combine the advantages of both techniques. Microarrays are considered powerful, large scale tools to examine thousands of genes and generate a list of candidate genes, whereas some might be novel and cannot be pointed out by small scale qRT-PCR experiments alone. Therefore, microarray data often provide the foundations for small scale qRT-PCR studies.

Table 7.3: Variations between the microarray and the qRT-PCR technologies used for the current research.

Feature	Affymetrix® microarray	qRT-PCR
Number of genes tested	Large	Small
Probe design	11 PM-MM probe-set design (Tian et al., 2004)	TaqMan® assay (Shippy et al., 2004)
cDNA amplification	Linear (IVT) (Van Gelder et al., 1990)	Exponential (Nolan et al., 2006)
Labelling	Direct (Do and Choi, 2007)	Indirect (Busten and Nolan, 2004)
Normalisation and calculation of gene intensity value	GCRMA normalisation (Wu et al., 2004)	$2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001)
Statistical analysis	Specialised data mining techniques	Standard (non-parametric) techniques

IVT, *in-vitro* transcription; GCRMA, G-C base pair robust multi-array average.

7.3. IDENTIFICATION OF CRANIOFACIAL PHENOTYPES

Craniofacial deformities exhibit a wide range of clinical, dental and radiographic phenotypes. Previous masseter muscle genotype-phenotype studies have used various phenotypic classifications. Most of these classifications were based on either vertical (Singh et al., 2000; Suchak et al., 2009) or horizontal (Gedrange, et al., 2005; Harzer et al., 2007) patterns, and none have assessed the effect of both dimensional discrepancies on masseter muscle genotype. Therefore, prior to the current genotype-phenotype correlation, it was necessary to establish the craniofacial morphology of the subjects. Using various classifications reported in the literature (Sassouni, 1969; Schendel et al., 1976), together with the analysis of the clinical, dental and radiographic features, four main phenotypic classifications were produced:

1. Basic vertical classification (long faces vs. controls –average vertical faces).

2. Basic horizontal classification (Class II vs. Class III vs. controls –average horizontal faces).

3. Combined vertical and horizontal classification (Class II long faces vs. Class III long faces vs. Class III average faces vs. controls –average vertical and horizontal faces).

4. Subdivisions of the long face classification (long faces with AOB vs. long faces without AOB vs. controls –average vertical faces).

The reason for generating four sets of phenotypic data was to assess whether the grouping of patients based on different craniofacial dimensional patterns would have an effect on the interpretation of the genotypic data. The current research demonstrated that basic vertical and horizontal classifications may not be sufficient for genotypic correlation.

This was evident when the “informative” *MYH* genes, as well as the “novel” genes ascertained from the present microarray experiment, showed no significant differences between patients, when grouped solely based on vertical facial development. While,

regrouping based on both vertical and horizontal patterns or using the long face subdivisions (with and without AOB), some of the "informative" and "novel" genes showed significant differences between various groups. This indicates the effect of both vertical and horizontal facial development on masseter muscle gene expression, and that these dimensional patterns should not be ignored during classification.

However, one has to acknowledge the difficulties of using detailed classifications which compromises the sample size, particularly invasive genetic studies, including the current research, where limited numbers of patients are often encountered (Gedrange et al., 2006; Suchak et al., 2009). Therefore, careful interpretation of the data is required and further investigation, using a large sample size with more coherent features of the different classified groups, is needed.

7.4. GENOTYPE-PHENOTYPE CORRELATION

7.4.1. INFORMATIVE MASSETER MUSCLE GENOTYPE IN RELATION TO CRANIOFACIAL PHENOTYPES

Out of the six "informative" myosin heavy chain genes (*MYH*1; *MYH*2; *MYH*3; *MYH*6; *MYH*7; *MYH*8), the *MYH*3, *MYH*6 and *MYH*7 have been found to be up-regulated in Class II patients compared to Class III individuals and the controls.

All of the Class II patients included in the current research exhibited a long face appearance with AOB. However, both *MYH*3 and *MYH*6 showed up-regulation in Class II patients when the recruited subjects were classified based on only horizontal facial features, while when the patients were regrouped using the various other classifications, no variations have been found. This may have been attributed to the large individual variations that were observed in the expression of the *MYH*3 and *MYH*6 genes (as shown by the box plots presented in Chapter 6, Figure 6.2).

*MYH*7 on the other hand, showed consistent up-regulation in relation to Class II appearance, Class II long face pattern and the long face with AOB group when using the various classifications. Furthermore, the present data indicate that the masseter muscle gene expression of the *MYH*7 was positively correlated to ANB angle. This suggests

that Class II individuals with severe retrognathic appearance (increased ANB) and long face pattern with AOB tend to have over-expression of the *MYH*7. The current results confirm the findings of Gedrange and co-workers, (2006) who reported up-regulation of *MYH*7 in Class II patients compared to Class III individuals.

Whether the gene expression of the *MYH*7 can be considered a predictor of a specific facial type or as an indicator for possible relapse following orthognathic surgery, would require extensive investigation on genomic, proteomic and transcriptomic levels, particularly before and after surgical correction. Harzer and colleagues, (2007) found a shift in *MYH*7 gene expression in Class II patients six months following mandibular advancement. However, long-term studies are required to establish whether the mode of the shift is transitional or permanent.

7.4.2. NOVEL MASSETER MUSCLE GENOTYPE IN RELATION TO CRANIOFACIAL PHENOTYPES

Out of the five "novel" masseter muscle genes, two new discoveries have been reported. Both SERGEF (Secretion Regulating Guanine nucleotide Exchange Factor) and NDRG2 (N-myc Downstream Regulated Gene family member 2) genes have been found to be down-regulated in relation to the Class III pattern.

7.4.2.1. NDRG2 masseter muscle gene expression

Although the gene expression status of the NDRG2 showed inconsistent results between the microarray (up-regulated in Class III compared to Class II) and qRT-PCR (down-regulated in Class III compared to Class II) data, the clinical, dental and radiographic analysis in relation to qRT-PCR gene expression has been found to be consistent. This was evident since the NDRG2 gene expression, as dictated by qRT-PCR, has been found to be down-regulated in relation to the general clinical appearance of the Class III pattern including both subgroups (Class III long face and Class III average face compared to Class II long face) and was positively correlated to the number of dental occlusal contacts, SNA and ANB angles.

NDRG2 has been found to be down-regulated in progressively dividing cancer cells compared to normally dividing cells (Zhao et al., 2008), while up-regulation was found

to suppress cancer cell proliferation (Kim et al., 2009). Down-regulation of NDRG2 gene in Class III patients may indicate the craving situation of masseter muscle cells to proliferate and regenerate as a result of the continuous micro trauma generated from the imbalanced relationship between masseter muscle and the underlying prognathic skeletal deformity.

7.4.2.2. SERGEF masseter muscle gene expression

Both the microarray and qRT-PCR results for the gene expression of SERGEF were consistent in relation to the clinical, dental and radiographic features of Class III individuals compared to Class II subjects and the controls. SERGEF gene expression was down-regulated in relation to a general prognathic clinical appearance, regardless of the vertical facial development in both Class III subgroups, without being affected by the fact that some of the Class III patients exhibited long face appearance while others had average vertical facial features. SERGEF gene expression was also positively correlated to the ANB angle and the number of dental occlusal contacts.

A possible cause for the involvement of SERGEF in the masseter muscle of Class III prognathic patients could be the reduced masseter muscle activity associated with the Class III pattern (Cha et al., 2007) and the reduced number of dental occlusal contatcts (Kobayashi et al., 2001). As a result, intracellular ion exchange is affected so that high levels of Ca^{2+} ions appear in fast-contracting muscles (Allen and Leinwand, 2002), while Mg^{2+} tend to increase in weak muscles (Dell Castillo and Engbaek, 1954). Increased Mg^{2+} levels promote binding of down-regulated SERGEF to an exocyst called Sec5 (Sjölinder et al., 2004). The exocysts are a group of proteins that can modulate the secretion and traffic pathway of various extracellular molecules (Kee et al., 1997). The compound of SERGEF attached to Sec5 has been found to modulate and increase the secretion but not the synthesis of chondroitin sulphate proteoglycans in cancer cell lines (Sjölinder et al., 2002). Chondroitin sulphate proteoglycan is one of the extracellular matrix proteins that is developmentally regulated (Velleman et al., 1999) and highly expressed during embryonic development of skeletal muscles. A marked reduction in expression has been observed in adults (Carrino, 1999; Velleman et al., 1999) except following injury (Carrino et al., 1988). This may suggest the need for the masseter muscle to regenerate in compensation for the imbalanced musculo-skeletal relationship

associated with a Class III pattern. However, these speculations need to be further investigated and proven prior to any conclusion. Figure 7.1 illustrates a hypothesised role of the SERGEF gene in the masseter muscle of Class III patients.

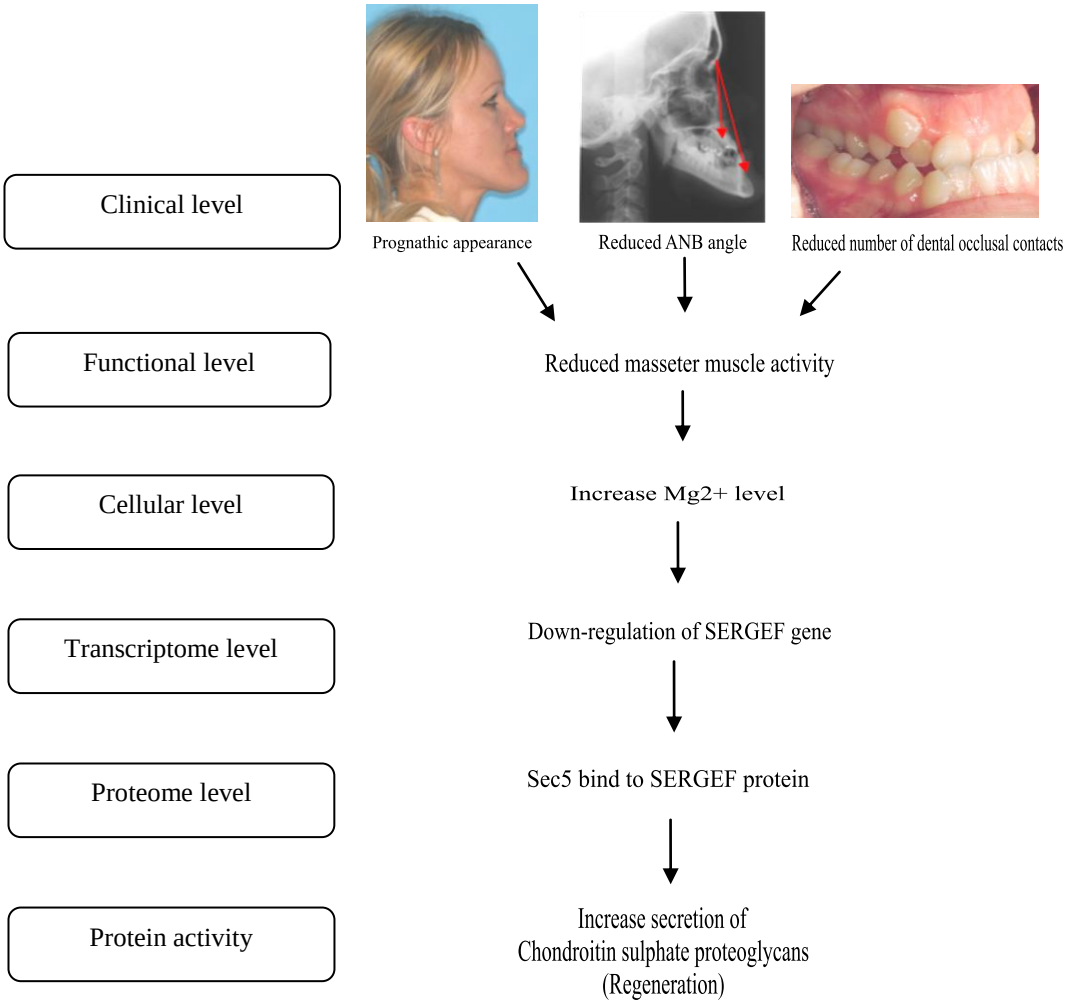


Figure 7.1: A hypothesised role of SERGEF gene in the masseter muscle of patients with a Class III pattern*. Weak masseter muscle activity associated with Class III appearance would increase the intracellular levels of Mg^{2+}, which would promote binding of down-regulated SERGEF to sec5 and subsequently induce masseter muscle regeneration via increasing the secretion of chondroitin sulphate proteoglycans.*

7.5. CONCLUSIONS

- The current research has provided an insight into the transcriptome profile of the masseter muscle and has identified new genes that affect musculo-skeletal morphology.

- By using microarray technology, foundation for the pre-treatment gene expression profile of the masseter muscle in relation to various craniofacial deformities has been set.

- The use of combined vertical and horizontal classifications is a better approach than either type of grouping alone in identifying the phenotype of craniofacial deformities in genotype-phenotype analysis.

- The greater the retrognathic appearance, the higher the *MYH*7 gene expression

- The greater the prognathic appearance, the lower the NDRG2 and SERGEF gene expression.

- Results including Class II subjects should be interpreted with caution since the facial heights were greater than normal.

7.6. SUGGESTIONS FOR FUTURE WORK

7.6.1. PATIENT CLASSIFICATION FOR MASSETER MUSCLE RESEARCH

- The use of a comprehensive patient classification including clinical, dental and radiographic criteria is recommended when assessing the masseter muscle genotype, whether on genomic, transcriptomic or proteomic levels. This would provide a precise genotype-phenotype correlation.

7.6.2. ON A GENOME LEVEL

- DNA sequencing of the SERGEF and NDRG2 genes of patients with variable craniofacial morphologies is recommended. This would enable an assessment as to whether the gene expression variations were due to mutations of the genes or a gene expression response to the skeletal discrepancy.

7.6.3. ON A TRANSCRIPTOME LEVEL

- Post-surgical follow-up of the masseter muscle gene expression in patients undergoing corrective surgery, preferably on a long-term basis including relapse cases using both microarray and qRT-PCR, is recommended. This would provide an insight into the behaviour of the genes, and whether or not any genes would demonstrate a transitional shift or permanent adaptation in response to the surgical correction, as well as the possibility of identifying certain genes that may be considered predictors for orthognathic surgical relapse.

- Gene expression microarray of the masseter muscle of both syndromic and non-syndromic patients having similar craniofacial patterns would provide valuable information regarding the mechanism of action and the various genetic pathways in syndromic and non-syndromic conditions. This could be combined with pre- and post-surgical assessment.

7.6.4. ON A PROTEOME LEVEL

- Future experimentation of the protein activity of the *MYH* and novel genes of the masseter muscle pre- and post-surgical correction of jaw deformities may

establish their role in relation to existing discrepancies and in response to jaw modification.

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Internet links

Figure 1.2: General muscle structure. Cited 2006 May 15, from: http://images.google.co.uk/imgres?imgurl=http://www.dkimages.com/discover/thumbs/961/50310869.JPG&imgrefurl=http://www.dkimages.com/discover/home/health-and-beauty/Human-Body/Muscle-System/Index.html&usg=__8BE9ZlSBGAT2BNYl-kMsqbBznx4=&h=120&w=120&sz=3&hl=en&start=158&tbnid=2gdrAyD56nd6iM:&tbnh=88&tbnw=88&prev=/images%3Fq%3Dmuscle%2Bfascicles%26gbv%3D2%26ndsp%3D20%26hl%3Den%26sa%3DN%26start%3D140

Figure 1.3: The motor unit in muscle fibres. Cited 2008 December 05, from: http://images.google.co.uk/imgres?imgurl=http://www.octc.kctcs.edu/GCaplan/anat/images/Image336.gif&imgrefurl=http://www.octc.kctcs.edu/GCaplan/anat/Notes/API%2520Notes%2520J%2520%2520Muscle%2520Contraction.htm&usg=__j3tt2q_t5lUSbqRISDJ4twZtPN0=&h=440&w=533&sz=30&hl=en&start=3&tbnid=KB1v1CnbwGavdM:&tbnh=109&tbnw=132&prev=/images%3Fq%3Dactin%2Bmyosin%26gbv%3D2%26hl%3Den

Microarray sample size estimation. Cited 2008 March 24 from: http://bioinformatics.mdanderson.org/MicroarraySampleSize/

Appendix A. Ethical papers

Eastman Dental Hospital ethical approval/UK -1 of 3 pages

NHS

The National Hospital for Neurology and Neurosurgery & Institute of Neurology Joint REC
1st Floor, Maple House
149 Tottenham Court Road
London W1P 9LL
POSTAL ADDRESS:
Ground Floor, Rosenheim Wing
25 Grafton Way
London
WC1E 5DB
Tel: 020 7380 9579
Fax: 020 7380 9937
Email: michael.gilberthorpe@uclh.nhs.uk
Website: www.uclh.nhs.uk

Professor Nigel P Hunt
Chairman of Division of Craniofacial Growth and Development and Head of Unit of Orthodontics /UCL Eastman Dental Institute
UCL Eastman Dental Institute and UCL Hospitals Trust
Eastman Dental Institute /Dept of Orthodontics
256 Gray's Inn Rd
London
WC1X 8LD

Our Ref: 06L 001

06 January 2006

Dear Professor Hunt

Full title of study: **A microarray study to assess genotypic and gene expression variation in maxillary and mandibular bone and masseter muscle in patients exhibiting variable craniofacial deformities undergoing orthognathic surgery**

REC reference number: **05/Q0512/120**

Thank you for your letter of 19 December 2005, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information was considered at the meeting of the Sub-Committee of the REC held on 05 January 2006. A list of the members who were present at the meeting is attached.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Ethical review of research sites

The Committee has agreed that site-specific assessment is not required for the following site(s):

The Eastman Dental Hospital ethical approval/UK cont -2 of 3 pages

Research site	*Name of PI (CTIMPs only) or local contact point*	*Post*
Eastman Dental Hospital/Eastman Dental Institute 256 Gray's Inn Road London WC1X 8LD	Professor N P Hunt	Chairman of Division of Craniofacial Growth and Development and Head of Unit Orthodontics UCL Eastman Dental Institute

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Application		19 October 2005
Investigator CV Professor N P Hunt	August 2005	
Investigator CV	Mark Peter Lewis	15 September 2005
Protocol	1.0	14 October 2005
Covering Letter		14 October 2005
Statistician Comments	Dr Jacky Pallas	15 September 2005
Letter of invitation to participant	1.0	14 October 2005
GP/Consultant Information Sheets Letter to GP	1.0	14 October 2005
Participant Information Sheet	2	19 December 2005
Participant Information Sheet Variation Group	1.0	14 October 2005
Participant Information Sheet Average Group	1.0	14 October 2005
Participant Information Sheet Leaflet	1.0	14 October 2005
Participant Consent Form	1.0	14 October 2005
Material & Methods flow chart	1	
Summary of Protocol	1.0	14 October 2005

Research governance approval

The study should not commence at any NHS site until the local Principal Investigator has obtained final research governance approval from the R&D Department for the relevant NHS care organisation.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

05/Q0512/120	**Please quote this number on all correspondence**

The Eastman Dental Hospital ethical approval/UK cont -3 of 3 pages

With the Committee's best wishes for the success of this project

Yours sincerely

pp. M. Cummins

Mrs Katy Judd
Chair

Email: michael.gilberthorpe@uclh.nhs.uk

Enclosures:

Standard approval conditions

Copy to: Research & Development Department – UCLH

Professor C Scully
Dean & Director of Studies and Research
Eastman Dental Institute

Ms H Moawad

Riyadh Military Hospital ethical approval/Saudi Arabia

Kingdom of Saudi Arabia
Ministry of Defense and Aviation
Chief of Staff
Medical Services Department

P.O. Box 7897 Riyadh 11159
Tel: 4777714 Fax: 4762121
Email: Info@rkh.med.sa

R.M.H.

مستشفى القوات المسلحة بالرياض
Riyadh Military Hospital

المملكة العربية السعودية
وزارة الدفاع والطيران والمفتشية العامة
رئاسة هيئة الأركان العامة
الإدارة العامة للخدمات الطبية للقوات المسلحة

ص.ب ٧٨٩٧ - الرياض ١١١٥٩
هاتف: ٤٧٧٧٧١٤ فاكس: ٤٧٦٢١٢١
بريد الكتروني : Info@rkh.med.sa.

RESEARCH & ETHICAL COMMITTEE

05 September 2006

Dr. Hadwah Moawad
Department of Dentistry

Re: A microarray study to assess genotypic and gene expression variation in maxillary and mandibular bone and masseter muscle in patients exhibiting variable craniofacial deformities undergoing orthognathic surgery

Dear Dr. Hadwah

I am pleased to inform you that at the recently held meeting (29 August 2006), the Research & Ethical Committee approved the above project with no cost to RKH Program.

Your research protocol has now been documented under:

Project No.	314
Minutes	06/004
Series of	2006

Kindly quote the project number indicated herein in all transactions and communications. You are advised to submit a report in relation to this research scheme to update the committee of its progress.

I trust your research scheme proves fruitful and beneficial to the RKH Program.

Yours sincerely

PROF. MOHAMMAD TARIQ, PhD FRCPath FRSC
Acting Chairman, Research & Ethical Committee
First floor, Building 136
Riyadh 11159, Saudi Arabia

Whipps Cross University Hospital ethical approval/UK

The National Hospital for Neurology and Neurosurgery
& Institute of Neurology Joint REC
Research & Development
1st Floor, Maple House
Ground Floor, Rosenheim Wing
25 Grafton Way
London
WC1E 5DB
Tel: 020 7380 9940
Fax: 020 7380 9937
Email: sasha.vandayar@uclh.nhs.uk
Website: www.uclh.nhs.uk

Professor Nigel P Hunt
Chairman of Division of Craniofacial Growth and
UCL Eastman Dental Institute and UCL Hospitals
Trust
Eastman Dental Institute /Dept of Orthodontics
256 Gray's Inn Rd
London
WC1X 8LD
Our Ref: 07L 010

15 January 2007

Dear Professor Hunt

Full title of study: **A microarray study to assess genotypic and gene expression variation in maxillary and mandibular bone and masseter muscle in patients exhibiting variable craniofacial deformities undergoing orthognathic surgery**

REC reference number: **05/Q0512/120**

The REC gave a favourable ethical opinion to this study on 05 January 2006.

Further notification(s) have been received from local site assessor(s) following site-specific assessment. On behalf of the Committee, I am pleased to confirm the extension of the favourable opinion to the new site(s). I attach an updated version of the site approval form, listing all sites with a favourable ethical opinion to conduct the research.

Research governance approval

The Chief Investigator or sponsor should inform the local Principal Investigator at each site of the favourable opinion by sending a copy of this letter and the attached form. The research should not commence at any NHS site until research governance approval from the relevant NHS care organisation has been confirmed.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

05/Q0512/120	**Please quote this number on all correspondence**

Yours sincerely

Sasha Vandayar
Committee Co-ordinator

Email: Sasha.Vandayar@uclh.nhs.uk

Enclosure: *Site approval form*

An advisory committee to London Strategic Health Authority

Statistician's letter -1 of 2 pages

Bloomsbury Centre for Bioinformatics
Dept. Computer Science
University College London
Gower Street
London
WC1E 6BT
Tel. 0207 679 7210
www.bcb.lon.ac.uk help@bcb.lon.ac.uk

15 September 2005

Dr. Mark Lewis
Eastman Dental Institute
University College London
256 Grays Inn Road
London WC1X 8LD

Dear Dr. Lewis

RE: A microarray study to assess genotypic and gene expression variation of the bones of the jaws and facial muscles of patients exhibiting variable craniofacial deformities undergoing jaw surgery

In answer to some of your questions about microarray data analysis and the number of biological replicates required for your proposed study I can make some observations and discuss best practice. However in the absence of any pilot study data it is difficult provide definite guidelines.

Your study involves sampling from two distinct groups of patients – control and deformity. In the deformity group you have indicated there are six sub-groups. Samples for microarray analysis will be obtained from the bone, muscle and epithelium of each patient in the study.

Microarray statistics is still a developing area of research. Analysis is difficult where the number of tests (eg genes on an array) is large and the number of samples small. Even a conservative p.value of 0.01 means that 1% of genes are false-positives. For a controlled in vitro experiment where the target p-value is 0.01 and the target log ratio is 2-fold, the number of required arrays is 6. This number is dependent on experimental data and assumes a normal distribution for the gene expression values. The number of replicates required is calculated from the equation:

Arrays = ((standard deviation X inverse normal distribution X (1-p.value)) / target log ratio)^2

For a target p-value of 0.05 and a target log ratio of 0.5 (1.4-fold) the number of required replicates is 11.

Statistician's letter cont -2 of 2 pages

There are two main sources of error in your proposed experiment:

Biological variation, from patient to patient. As each patient will have differing genotypes this will impact the study. Therefore the recommendation is for increased numbers of patients to minimise genotype effects and sample variation.

Technical variation, from array to array. As the samples will be collected and hybridised to GeneChips over a considerable time period, the technical variation becomes significant – batch changes, reagent and experimenter changes are all sources of error. The technical variation can be accounted for with increased replication.

Therefore I suggest that you have, if possible, at least 10 patients per subgroup, and at least 10 control patients.

We routinely use the BioConductor suite of packages to analyse microarray data. These are algorithms written in the R statistical programming language by experts in the statistical analysis of microarraydata (http://www.bioconductor.org/; Gentleman et al., 2004). GeneChip quality and experimental reproducibility are assessed using the affy package. Probe data is normalized using GCRMA (Wu et al., 2004) and MAS5.0 algorithms. Genes which show significant differential expression are identified using the Linear Models (limma) package (Smyth, 2004). Subsequent clustering of significant genes will be tested with bootstrapping.

Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JYH, Zhang J (2004) Bioconductor: open software development for computational biology and bioinformatics. Genome Biology 5: R80

Smyth GK (2004) Linear Models and empirical Bayes methods for assessing differential expression in microarray experiments. Stat Appl Gen Mol Biol 3: No. 1, Article 3

Wu Z, Irizarry RA, Gentleman R, Martinez-Murillo F, Spencer F (2004) A model-based background adjustment for oligonucleotide expression arrays. J Am Stat Assoc 99: 909-917

I hope that this addresses some of the questions you have about experimental design and replication. Please do not hesitate to contact me if you have further questions.

Sincerely,

Dr. Jacky Pallas
Scientific Manager
Bloomsbury Centre for Bioinformatics

Invitation letter

Version 1.0
Date: 14/10/2005

Study title: A genetic study of facial /masticatory muscle and bones of the jaws in patients with variable facial features.

You are kindly invited to take part in the above mentioned study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the information sheet and leaflet supplied with this letter and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information.

Thank you for taking the time to read this.

For further information please contact any of the Researchers listed below.

- Professor Nigel Hunt

Chairman of Division of Craniofacial and Developmental Sciences, and Head of Units of Orthodontics and Paediatric Dentistry.
UCL Eastman Dental Institute.
256 Grays Inn Road
London WC1X 8LD
Phone: +44 (0) 20 7915 1239
E-Mail: n.hunt@eastman.ucl.ac.uk

- Miss Hadwah A.M Moawad

PhD postgraduate, Unit of Orthodontics
UCL Eastman Dental Institute
256 Grays Inn Road
London WC1X 8LD
Phone: +44 (0) 20 7915 1054
E-Mail: h.moawad@eastman.ucl.ac.uk

Participant's information sheet -1 of 5 pages

This information sheet was supplied for both the deformity and control groups. However, questions number 4, 6 and 7 were modified for the control group.

Version: 2.0
Date: 19/12/2005
Project ID:

1. Study title
A genetic study of facial /masticatory muscle and bones of the jaws in patients with variable facial features.

2. Invitation
You are kindly invited to take part in this study, which will provide valuable genetic information about the muscle-bone interaction to layout facial features. Before you decide, it is important for you to understand why the research is being conducted and what it will involve. Please take time to read this information sheet as well as the leaflet given carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether you wish to take part or not.

3. What is the purpose of the study?
Previous studies have shown that both bone and facial muscles contribute to the development of facial features. However, the question as to whether the shape of the bones of the jaws are affected by the development of facial muscles or that the muscles adapt to the underlying bone remains unanswered. Therefore, this study will investigate the genetic variation of the bones of the jaws and facial muscles of patients with variable facial features compared to individuals with average facial form. Furthermore, orthodontic /jaw surgery treatment modifies the shape of the bones of the jaws which gives an opportunity to assess the effect of changing the underlying bone on the adaptability of facial muscles.

Participant's information sheet cont -2 of 5 pages

4. Why have I been chosen?

A clinical examination has shown that you have a variation in your jaw form which you have agreed to correct surgically. Therefore, you are invited to take part in this research which will include 2 main groups (average and variation group). You are considered one out of many other patients within the variation group.

5. Do I have to take part?

Participation in this study is completely voluntary and up to you. If you decided to take part, you will be given this information sheet as well as a leaflet to keep and will be asked to sign a consent form. If you decide to withdraw, you are free to do so at any time without giving a reason. A decision to withdraw or not to take part in the study will not affect your care and treatment at the Eastman Dental Hospital in any way.

6. What is involved in the study?

Taking part in this research will not require any additional appointments other than the appointed time for orthodontic /jaw surgery treatment. Three different tissue samples (an oral swab, muscle biopsy and the remaining bone fragments) will be required. The remaining bone fragments will be collected only following the removal of your wisdom teeth and jaw surgery. The oral swab and the muscle biopsy will be collected from the inner cheek at three different appointments spread over the entire treatment. The three appointments are:

At the time of removal of the wisdom teeth.

At the time of jaw surgery.

At least 6 months after surgery.

7. What are the procedures being conducted?

The oral swab is a simple procedure done by rubbing the inner cheek with a special brush. As for muscle samples, at the first two appointments, general anaesthesia will be administered for the surgical procedures. Therefore, no additional anaesthesia is required. On the third appointment, the biopsy will be conducted under local anaesthesia following a routine orthodontic appointment, which would require about 10

Participant's information sheet cont -3 of 5 pages

minutes extra than the appointed time for orthodontic treatment. As for bone, only the remaining fragments following surgery will be collected.

8. What are known risks of the study or the side effects of any procedure conducted?
No complication or side effects are expected from collecting the remaining bone fragments or the oral swab. As for the muscle sample, over the last 5 years, several other studies at the Eastman Dental Hospital have collected muscle biopsies from a large number of patients with no reported complications. However, you may feel slight discomfort or oozing of blood following the biopsy. Therefore, to ensure maximum comfort and safety, your general practitioner will be informed about the study and the time when the samples will be collected.

9. What are the possible benefits of taking part?
The information we get from this study may help us as professionals to improve the quality of treatment provided for future patients to last for a lifetime.

10. The information held about the research subject
All collected samples will be stored at the UCL/ Eastman Dental Institute and used for genetic analysis for this study only. All the information collected for this research will be strictly confidential. In order to maintain confidentiality, once collected, the samples will be given an identifier code (eg. NBS 1 i.e., normal bone sample from patient number one) purely to relate the data. Therefore, all personal details (name, age, etc) will not be required and will only be known by the principle investigators.

11. Studies on tissue
We are very grateful to all subjects that the oral swab, muscle biopsy and the remaining bone fragments will be donated as a gift which will be used in this study only. Once the research is finished, all samples will be destroyed.

Participant's information sheet cont -4 of 5 pages

12. Gene studies

This study was designed to identify genes involved in bone development and muscle adaptability. Therefore, any identified genes will be related to facial features rather than the patient's general health status. Furthermore, the genetic analysis will be linked to one group (either the average or the variation group) rather than to a single patient.

13. What happens when the research study stops?

All samples will be destroyed when the study is finished.

14. What will happen if the findings may affect the subject personally?

It is unlikely that the results of the study will affect any of the subjects personally. If any of the patients are interested to know the end results, it could be arranged to send them a copy whenever the study is published.

15. What if something goes wrong?

If you have any comments or concerns you may discuss these with any of the researchers. If you wish to go further and complain about any aspect during the course of the research you may do so. You should contact the Complaints Manager, UCL Hospital. Please Quote the UCLH project number at the top of this information sheet.

16. What will happen to the results of the research study?

The results of this study will be published in scientific journals. However, none of the patients will be identified in any way. The genetic and facial feature comparison will be purely between groups (variation and average) rather than individuals. If you are interested in finding out the final results, please let us know then we could either send you an abstract of the published paper or the name of the journal and the date of publication.

17. Who is organising and funding the research?

This study is part of a postgraduate research programme sponsored by the University College London/ Eastman Dental Institute.

Participant's information sheet cont -5 of 5 pages

18. Inducements

Participation in this research will not affect your care and treatment at the Eastman Dental Institute in any way.

19. Withdrawal from the project

Your participation in the trial is entirely voluntary. You are free to decline to enter or to withdraw form the study any time without having to give a reason. If you choose not to enter the trial, or to withdraw once entered, this will in no way affect your future dental care. All information regarding your medical records will be treated as strictly confidential and will only be used for medical purposes. Your medical records may be inspected by competent authorities and properly authorised persons, but if any information is released this will be done in a coded form so that confidentiality is strictly maintained. Participation in this study will in no way affect your legal rights.

20. Who has reviewed the study?

This study has been reviewed by one of the UCLH Research Ethics Committees/ The National Hospital for Neurology and Neurosurgery and Institute of Neurology Joint REC.

21. Contact for further information

If you have any questions at any time, please feel free to contact any of the researchers listed below.

- Professor Nigel Hunt
- Miss Hadwah A.M Moawad

Thank you for your cooperation

Consent form

Version 1.0
UCLH Study ID number: 05/Q0512/120
Date: 14/10/2005

Study title: A genetic study of facial /masticatory muscle and bones of the jaws in patients with variable facial features.

Name of Researchers: Professor Nigel Hunt and Miss. Hadwah A.M Moawad

- I confirm that I have read and understand the information sheet and leaflet for the above study and have had the opportunity to ask questions.
- I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.
- I understand that sections of my medical notes may be looked at by responsible individuals from the Eastman Dental Hospital. I give permission for these individuals to have access to my records.
- I give permission for the Researchers to collect tissue samples from the inner cheek, bones of the jaws and masseter muscle to conduct gene analysis for this study only.

I agree to take part in the above study.

……..	……	……..
Name of patient	Date	Signature
……..	……	……..
Name of person taking consent (If different from Researcher)	Date	Signature
……..	……	……
Name of Researcher	Date	Signature

1 copy for patient; 1 for researcher; 1 to be kept with hospital notes

Patient information Leaflet

Attached as a separate document to the back cover of the thesis.

Appendix B. Materials

Materials used for handling masseter muscle biopsy

Experimental step	Materials	Supplying company
Sample collection	-RNA*later*® tissue protect tubes	-Qiagen, West Sussex, UK
Dissection	-Vanna's microscissors, straight -Springbow dissecting scissors, extra fine, straight -Dumont tweezers, stainless steel	-Agar Scientific Ltd, Stansted, UK
Weighing of the sample	-Electronic balance Precisa 1600C	-Precisa balancers Ltd, Milton Keynes, UK

Materials used for RNA extraction and quality control

Experimental step	Materials	Supplying company
RNase inhibition		
-Clean surfaces	-RNase*Zap*® wipes -RNase® Zap spray	-Ambion (Europe) Ltd, Applied Biosystems, Warrington, UK
-Tips	-Eppendorf® epT.I.P.S,	-Sigma-Aldrich Ltd, Dorset, UK.
-Centrifuge tubes	-RNase/ DNase free centrifuge tubes	-Qiagen, West Sussex, UK
-Water	-RNase free water	-Qiagen, West Sussex, UK
Disruption and homogenisation		
-Lysing matrix beads	-x2ml Lysing Matrix D	-Q-BIOgene, Cambridge, UK
-Crushing machine	-FastPrep 120	-Q-BIOgene, Cambridge, UK
Purification and elution		
-selective binding	-Ethanol	-Fisher Scientific UK Ltd, Loughborough, UK
-Elusion	-RNeasy® mini kit	-Qiagen, West Sussex, UK
-DNase digestion	-RNase-free DNase set	-Qiagen, West Sussex, UK
RNA quantity and purity		
-Spectrophotometer	-Ultrospec 2000 machine	-Amersham Pharmacia Biotech, GE Healthcare Ltd, Buckinghamshire, UK
QC using the Bioanalyser		
-Preparation of gel dye mix	-Bioanalyser 6000 Nano kit	-Agilent Technologies Ltd, West Lothian, UK
-Loading the chip	-Priming station -Chip vortex (Nano chip was used)	-Agilent Technologies Ltd, West Lothian, UK
-Loading into the Bioanalyser	-RNeasy Zap solution	-Ambion (Europe) Ltd, Applied Biosystems, Warrington, UK
	-RNase free water	-Qiagen, West Sussex, UK
	-Agilent Bioanalyser 2100 machine	-Agilent Technologies West Lothian, UK
	-G2938A CellPro 2100 Bioanalyser Chip Reader v 1.4	-Agilent Technologies West Lothian, UK

QC: Quality control.

Materials used for Microarray laboratory work

Experimental step	Materials	Supplying company
Sample preparation	-GeneChip® Expression Eukaryotic Poly-A RNA Control kit	*
cDNA/cRNA synthesis and amplification	-GeneChip® Expression 3'-Amplificatoin Tow-Cycle cDNA Synthesis kit -MEGAscrip®T7 kit (for IVT)	* - Ambion (Europe) Ltd, Applied Biosystems, Warrington, UK
Synthesis of Biotin-labelled cRNA	-GeneChip® Expression 3'-Amplificatoin GeneChip IVT Labelling kit	*
cRNA fragmentation	-GeneChip® Sample cleanup module	*
Hybridisation	-GeneChip® Eukaryotic hybridisation control kit -GeneChip® Hybridisation oven 640	* *
Washing and staining	-GeneChip® Operating software (GCOS) v1.4 -GeneChip® Fluidics station 450	* *
Data extraction (scanning)	-GeneChip® Scanner 3000 -GCOS	* *
General materials	-GeneChip® Sample Cleanup Module -Bioanalyser 6000 Nano kit + Nano chip	* -Agilent Technologies Ltd, West Lothian, UK

*Supplied by Affymetrix UK Ltd, High Wycombe, UK.

Materials used for quantitative RT-PCR (qRT-PCR)

Experimental step	Materials		Supplying company
cDNA synthesis	-High-capacity cDNA reverse transcription kit - PTC-100 Thermocycler machine,		* -MJ Research Inc, Genetic research instrumentation Ltd, Essex, UK
Genes of interest	Assay ID number	GenBank mRNA	
-*MYH*1	Hs00428600_m1	AF111785.1 BC114545.1	*
-*MYH*2	Hs00430042_m1	AF111784.1 BX510904.2 BC1264409.1	*
-*MYH*3	Hs00159463_m1	X13988.1 BP232245.1 BQ956249.1	
-*MYH*6	Hs00411908_m1	D00943.1 BC117511.1 BC132667.1	* *
-*MYH*7	Hs00165276_m1	M58018.1 X51591.1 AY518538.1 AB209708.1 DQ248310.1 BC112173.1 BC112171.1 EF560725.1 EU747717.1	*
-*MYH*8	Hs00267293_m1	X51592.1 Z38133.1 M36769.1 AK303395.1	*
-LOC gene	Custom TaqMan® gene expression assya	BE675108.1	*
-SERGEF	Hs00183730_m1	AJ243950.1 AJ243951.1 BC000707.2 BC065375.1 AK292286.1	*
-KIAA1671	Hs01369792_m1	AL832019.1 BC171801.1	*
-DGCR6	Hs00606390_mH	X96484.1 AF228707.1 GR456434.1 BC047039.1 AK098780.1 AK35115.1	*
-NDRG2	Hs00212263_m1	AF159092.3 AW163815.1 AK024521.1 CR596981.1 CR597040.1 CR598346.1 CR601715.1 CR607901.1 CR611368.1 BC093038.1 AF087872.1 BX247987.1 BX647748.1 BI759006.1 AF304051.1 AK096999.1	*
qRT-PCR	-File builder software (to design LOC gene assay) -TaqMan® universal PCR master mix -MicroAmp® optical 96-well reaction plates -MicroAmp® optical adhesive cover -7300 Real time PCR machine* -7300 system software* -Large centrifuge		* * * * * * -Jouan CR4.12, DJB labcare Ltd, Buckinghamshire, UK.

Hs: Homo sapiens. _m1: An assay which spans an exon junction and will not detect genomic DNA. _mH: The assay is very sensitive as it was designed to a transcript belonging to a gene family with high sequence homology. *Supplied by Applied Biosystems, Warrington, UK.

Appendix C. Protocols

Final RNA extraction protocol

Laboratory steps	Materials	Volume	Settings
i) Prior to start Clean the working bench, pipettes and all plastic ware used	-RNase*Zap*® wipes -RNase*Zap* spray		
ii) Handling the sample -Cutting the muscle biopsy in the RNA*later*® tube (30mg)	Microscissors Tweezers		
iii) Disruption and homogenisation	Muscle biopsy Lysing matrix D RLT buffer Measure lysate 70% ethanol Total	~30mg 600µl ~450 µl ~450 µl * ~900 µl	FastPrep® homogeniser 1st round Speed 6 20s Cool on ice 5m 2nd round Speed 6 20s Cool on ice 5m Centrifuge 5m 10,000 rpm.
iv) Purification** -Use the lysate from the previous step	Spin column RW1 buffer DNase I mix**** RW1 buffer RPE buffer RPE buffer New tube open cap	~900 µl 350µl 80µl 350µl 500 µl 500 µl -	Centrifuge 15s 10,000 rpm*** Centrifuge 15s 10,000 rpm Keep 15m at room T Centrifuge 15s 10,000 rpm Centrifuge 15s 10,000 rpm Centrifuge 2m maximum speed Centrifuge 1m maximum speed
v) Elution** RNase free water	**30 µl**	Keep 5m at room T Centrifuge 1m maximum speed	

*Measure the remaining lysate after homogenization and add equal volume of ethanol. ** Following the RNeasy® mini kit manual. ***Capacity of the spin column is only 700 µl, hence the spinning can be conducted more than once. ****Using the RNase-free DNase set manual. T, temperature.

Agilent Bioanalyser Nano chip laboratory protocol

Laboratory steps	Materials	Volume	Settings
i) Preparation of the gel dye mix			
	RNA gel matrix	65µl	
	Blue dye	1µl	
Total	**Gel dye mix**	**66µl**	
ii) Loading the chip			Priming station
-Place the Nano chip in the priming station.			-Plunger at 1ml
-Add gel dye mix to the well marked G	Gel dye mix	9µl	-Press the plunger and hold for 30s then release to 1ml
-Add to all remaining 12 wells in the Nano chip	Gel dye mix	9µl	
-Add to wells being used + ladder	Marker	5µl	
-Add to blank wells	Marker	6µl	
-Load into the ladder well	Ladder	1µl	
-Load into the samples wells	RNA samples	1µl	Vortex 1m
iii) Cleaning the Bioanalyser			2100 Bioanalyser machine
-Load the Zap clear chip, then remove	RNeasy Zap solution	350µl	Load 1m
-Load the RNase H2O clear chip, then remove	RNase free water	350µl	Load 10s
-Leave the led open to dry			10s
iv) Loading the Bioanalyser			2100 Bioanalyser machine
-Load the 6000 Nano chip and close the lid	-	-	Use the 2100 software to operate and read the chip

Microarray laboratory protocol

Laboratory steps	Materials	Volume	Settings
i) Target preparation			
-Spike in Poly-A controls	diluted poly-A RNA	0.2µl	
	controls	0.2µl	
-Add Oligo(dt)	Concentrated T7-Oligo(dT)	1.6µl	
	RNase free water	Variable	
	RNA sample (total concentration **100ng**)	Variable	
	RNase free water	Variable	
Total	**Mixture 1**	**5µl**	
ii) cDNA/cRNA synthesis			Heating blocks
1st cycle, 1st strand cDNA	Mixture 1	5µl	70°C 6m
			4°C*
-1st cycle, 1st strand MM	5X 1st strand reaction mix	2µl	
	DTT (0.1M)	1µl	
	RNase inhibitor	0.5µl	
	dNTP (10mM)	0.5µl	
	SuperScript II.	1µl	
	Mixture 1	5µl	
Total	**Mixture 2 (1st strand cDNA)**	**10µl**	42°C 1h
			70°C 10m
			4°C*
1st cycle 2nd strand cDNA			
-1st cycle 2nd strand MM	RNase H	0.2µl	
	E.coli DNA polymerase I	0.6µl	
	dNTP (10mM)	0.4µl	
	RNase free water	4.8µl	
	Freshly diluted $MgCl_2$	4µl	
	Mixture 2	10µl	
Total	**Mixture 3 (ds-cDNA)**	**20µl**	16°C 2h
			75°C 10m
			4°C hold*
IVT			
-IVT MM	10X reaction buffer	5µ	
	Enzyme mix	5µ	
	ATP solutions	5µ	
	CTP solutions	5µ	
	UTP solutions	5µ	
	GTP solutions	5µ	
	Mixture 3	20µl	
Total	**Mixture 4 (cRNA)**	**50µl**	37°C 16h (overnight)
cRNA cleanup	RNase free water	50µl	
	IVT cRNA binding buffer	350µl	
	Ethanol (96-100%)	250µl	
	Mixture 4	50µl	
	Cleanup spin column	700µl	Centrifuge 15s ≥ 10,000 rpm.
	IVT cRNA wash buffer	500µl	Centrifuge 15s ≥ 10,000 rpm.
	80% Ethanol	500µl	Centrifuge 15s ≥ 10,000 rpm.
	Dry off the membrane		Centrifuge 5m at maximum speed
	RNase free water	13µl	Centrifuge 1m at maximum speed
Average	**Mixture 5 (cRNA elute)**	**11µl**	

Laboratory steps	Materials	Volume	Settings
Q.C of cleaned cRNA			
-Biophotometer	Mixture 5	2µl	-Eppendorf biophotometer
	RNase free water	78µl	
		80µl	
- Agilent Nano kit protocol	Mixture 5	1µl	-2100 Agilent Bioanalyser
			Acceptable A260/A280 = 1.8-2.29
2nd cycle, 1st strand cDNA	Freshly diluted random primers	2µl	
	Adjusted mixture 5 (total concentration **600ng**)	Variable	
	RNase free water	Variable	
Total	**Mixture 6**	**11µl**	70°C 10m
			4°C*
-2nd cycle, 1st strand MM	5X 1st strand reaction mix	4µl	
	DTT (0.1M)	2µl	
	RNase inhibitor	1µl	
	dNTP (10mM)	1µl	
	SuperScript II	1µl	
	Mixture 6	11µl	
Total	**Mixture 7 (1st strand cDNA)**	**20µl**	42°C 1h
			4°C*
	Mixture 7	20µl	
	RNase H	1µl	
Total	**Mixture 8**	**21µl**	37°C 20m
			95°C 5m
			4°C*
2nd cycle, 2nd strand cDNA	Freshly diluted T7 Oligo-dT	4µl	
	Mixture 8	21µl	
Total	**Mixture 9**	**25µl**	70°C 6m
			4°C*
-2nd cycle, 2nd strand MM	RNase free water	88µl	
	5X 2nd strand reaction mix	30µl	
	dNTP (10mM)	3µl	
	E.coli DNA polymerase I	4µl	
	Mixture 9	25µl	
Total	**Mixture 10 (ds-cDNA)**	**150µl**	16°C 2h
	T4 DNA polymerase	2µl	
	Mixture 10	150µl	
Total	**Mixture 11 (blunt-ended ds-cDNA)**	**152µl**	16°C 10m
			4°C*
ds-cDNA cleanup	cDNA binding buffer	600µl	
	Mixture 11	152µl	
	Cleanup spin column		Centrifuge 1m ≥ 10,000 rpm
	cDNA wash buffer	750µl	Centrifuge 1m ≥ 10,000 rpm
	Dry off the membrane		Centrifuge 5m at maximum speed
	cDNA elution buffer	14µl	Incubate 1m Room T, then
			Centrifuge 1m ≥ 10,000 rpm
Average	**Mixture 12 (ds-cDNA elute)**	**12µl**	
iii) cRNA synthesis and labeling			
	Mixture 12	12µl	
	10X IVT labelling buffer	4µl	
	IVT labelling NTP mix	12µl	
	IVT labelling enzyme mix	4µl	
	RNase free water	Variable	
Total	**Mixture 13 (labeled cRNA)**	**40µl**	37°C 16h (overnight)

Laboratory steps	Materials	Volume	Settings
Labeled cRNA cleanup	RNase free water	60µl	
	IVT cRNA binding buffer	350µl	
	Ethanol (96-100%)	250µl	
	Mixture 13	40µl	
	cRNA cleanup spin column	700µl	Centrifuge 15s ≥ 10,000 rpm.
	IVT cRNA wash buffer	500µl	Centrifuge 15s ≥ 10,000 rpm.
	80% Ethanol	500µl	Centrifuge 15s ≥ 10,000 rpm.
	Dry off the membrane		Centrifuge 5m at maximum speed
	RNase free water (1st elute)	11µl	Centrifuge 1m at maximum speed
	RNase free water (2nd elute)	10µl	Centrifuge 1m at maximum speed
Average	**Mixture 14 (final elute, cleaned and labeled cRNA)**	**21µl**	
Q.C of labeled cRNA			
-Biophotometer	Mixture 14	2µl	-Eppendorf biophotometer
	RNase free water	78µl	
		80µl	
-Agilent Nano kit protocol	Mixture 14	1µl	-2100 Agilent Bioanalyser Acceptable A260/A280 = 1.8-2.29
iv) cRNA fragmentation	Mixture 14 (total concentration **25µg**)	Variable	
	5X fragmentation buffer	8µl	
	RNase free water	Variable	
Total	**Mixture 15 (35-200 base fragmented cRNA)**	**40µl**	94°C 35m
Q.C of fragmented cRNA			
-Agilent Nano kit protocol	Mixture 15	1µl	-2100 Agilent Bioanalyser
v) Hybridisation			49 Format (Standard)/ 64 Format Array
-Hybridisation cocktail	Mixture 15 (total concentration **15µg**)	Variable	
	Control Oligonucleotide B2	5µl	
	20X Eukaryotic hybridisation controls (bioB, bioC, bioD, cre)	15µl	
	Herring sperm DNA (10mg/ml)	3µl	
	BSA (50mg/ml)	3µl	
	2X hybridisation buffer	150µl	
	DMSO	30µl	
	RNase free water	Variable	Hybridisation oven
Total	**Mixture 16 (hybridisation cocktail)**	**300µl**	99°C 5m
-Preparation of the chip	2X hybridisation buffer	150µl	45°C 10m (with rotation)
-Loading the array chip	Remove the 2X hybridisation buffer and add mixture 16	200µl	45°C 16h (60 rpm rotation)
vi) Washing and staining	Remove mixture16		Fluidics Station 450/250
1st wash	Non-stringent wash (Buffer A)	250µl	25°C 10 cycles 2 mixes
2nd wash	Stringent wash (Buffer B)	250µl	50°C 4 cycles 15 mixes
-Preparation of SAPE staining solution	2X stain buffer	600µl	
	BSA (final concentration 2mg/ml)	48µl	
	SAPE (final concentration 10µg/ml)	12µl	
	DI H_2O	540µl	
Total	**Mixture 17 (SAPE solution)**	**1200µl**	

Laboratory steps	Materials	Volume	Settings	
-Preparation of Antibody staining solution	2X stain buffer	300µl		
	BSA (final concentration 2mg/ml)	24µl		
	Goat IgG stock (final concentration 0.1mg/ml)	6µl		
	Biotinylated antibody (final concentration 3µg/ml)	3.6µl		
	DI H_2O	266.4µl		
Total	**Mixture 18 (Antibody solution)**	**600µl**		
1st stain	Mixture 17	600µl	25°C	10m
Post stain wash	Buffer A	250µl	25°C	10 cycles 4mixes
2nd stain	Mixture 18	600µl	25°C	10m
3rd stain	Mixture 17	600µl	25°C	10m
Final wash	Buffer A	250µl	30°C	15 cycles 4mixes

*Cooling down is at least for 2m. MM: Master mix. T: Temperature.

qRT-PCR laboratory protocol

Laboratory steps	Materials	Volume	Settings
i) cDNA synthesis			
-Adjusting RNA concentration (equal volume to the cDNA MM)	RNA sample (total concentration **5ng/reaction**)	Variable	
	RNase free water	Variable	
Total	**Adjusted RNA sample**	**10µl**	
-Preparation of cDNA MM	10X RT Buffer	2µl	
	25X dNTP mix (100mM)	0.8µl	
	10X RT Random primers	2µl	
	MultiScribe Reverse transcriptase enzyme	1µl	
	RNase free water	4.2µl	
Total	**cDNA MM/reaction**	**10µl**	
-cDNA synthesis	cDNA MM	10µl	T/ cycle (35 cycles used)
	Adjusted RNA sample	10µl	25°C 10m
Total	**cDNA mixture**	**20µl**	37°C 120m
			85°C 5s
			4°C ∞
ii) qRT-PCR			
-Preparation of qRT-PCR mix	TaqMan Universal PCR MM	12.5µl	
	RNase free water	8.75µl	
	cDNA mixture	2.5µl	
	Gene of interest assay	1.25µl	
Total	**qRT-PCR mixture/well**	**25µl**	
-Loading each well andcover the whole plate	qRT-PCR mixture	25µl	Centrifuge 15s maximum speed in a large centrifuge
-Applying the 96-well plate into the relative quantification real-time PCR machine			T/ cycle (40 cycles used)
			50°C 2m
			95°C 10m
			95°C 15s
			60°C 1m

MM: Master mix. T: Temperature.

Appendix D. Microarray: Quality control and summary of the project according to MIAME guidelines

GeneChip® quality control, values for each chip

Chip	Ploy-A controls	3'/5' ratio GAPDH	3'/5' ratio ß-actin	Hybridisation controls	% gene present	Back ground	Raw noise	Scaling factor
1 (R)	*Lys* 137.7 *Phe* 112.52 *Thr* 231.77 *Dap* 525.56	1.43	1.16	*BioB* 235.6 *BioC* 621.73 *BioD* 2567.42 *Cre* 8129.5	31	86.18	2.73	1.755
2 (R)	*Lys* 147.37 *Phe* 191.53 *Thr* 357.26 *Dap* 970.5	1.37	1.05	*BioB* 257.63 *BioC* 771.49 *BioD* 3217.92 *Cre* 9847.17	34.02	62.94	2.02	1.932
3	*Lys* 120.2 *Phe* 129.55 *Thr* 249.18 *Dap* 638.63	1.52	1.42	*BioB* 235.03 *BioC* 648.41 *BioD* 2481.85 *Cre* 8269.92	38	65.98	2.17	1.483
4	*Lys* 204.88 *Phe* 190.56 *Thr* 342.76 *Dap* 970.41	1.59	0.86	*BioB* 276.69 *BioC* 753.86 *BioD* 3268.68 *Cre* 10060.08	32.4	59.15	1. 92	2.379
5	*Lys* 145.77 *Phe* 124.74 *Thr* 244.07 *Dap* 767.22	1.46	2.21	*BioB* 133.38 *BioC* 131.01 *BioD* 1460.4 *Cre* 5292.6	47.0	57.82	2	0.596
6	*Lys* 142.09 *Phe* 143.11 *Thr* 374.07 *Dap* 1006.99	2.42	3.0	*BioB* 161.81 *BioC* 146.57 *BioD* 1649.44 *Cre* 6967.69	44.3	77.57	2.53	0.903
7	*Lys* 123.14 *Phe* 110.01 *Thr* 315.89 Dap 874.97	1.77	3.27	*BioB* 133.8 *BioC* 130.42 *BioD* 1377.26 *Cre* 6233.56	46.6	66.89	2.17	0.818
8	*Lys* 143.37 *Phe* 108.46 *Thr* 252.64 *Dap* 669.2	1.67	3.34	*BioB* 135.9 *BioC* 115.31 *BioD* 1326.16 *Cre* 2664.72	45.7	71.94	2.27	0.998
9	*Lys* 130.08 *Phe* 90.81 *Thr* 236.19 *Dap* 698.28	1.85	1.94	*BioB* 160.25 *BioC* 143.53 *BioD* 1631.71 *Cre* 7399.14	41.6	68.61	2.2	1.082
10	*Lys* 155.35 *Phe* 149.53 *Thr* 273.91 *Dap* 838.54	**3.09**	**6.22**	*BioB* 166.41 *BioC* 174.22 *BioD*1991.18 *Cre* 7639.73	50.9	64.54	2.09	0.864
11	*Lys* 129.33 *Phe* 92.65 *Thr* 262.38 *Dap* 902.49	1.67	2.75	*BioB* 134.67 *BioC* 114.83 *BioD* 1605.79 *Cre* 5873.5	46.9	69.13	2.15	0.696
12	*Lys* 94.28 *Phe* 61.12 *Thr* 127.53 *Dap* 393.68	1.43	1.76	*BioB* 152.57 *BioC* 139.74 *BioD* 1809.45 *Cre* 7093.53	43.7	67.19	2.12	0.865
13	*Lys* 341.06 *Phe* 332.59 *Thr* 757.38	1.58	2.03	*BioB* 156.24 *BioC* 167.31 *BioD* 1860.27	42.9	63.37	2.01	0.871

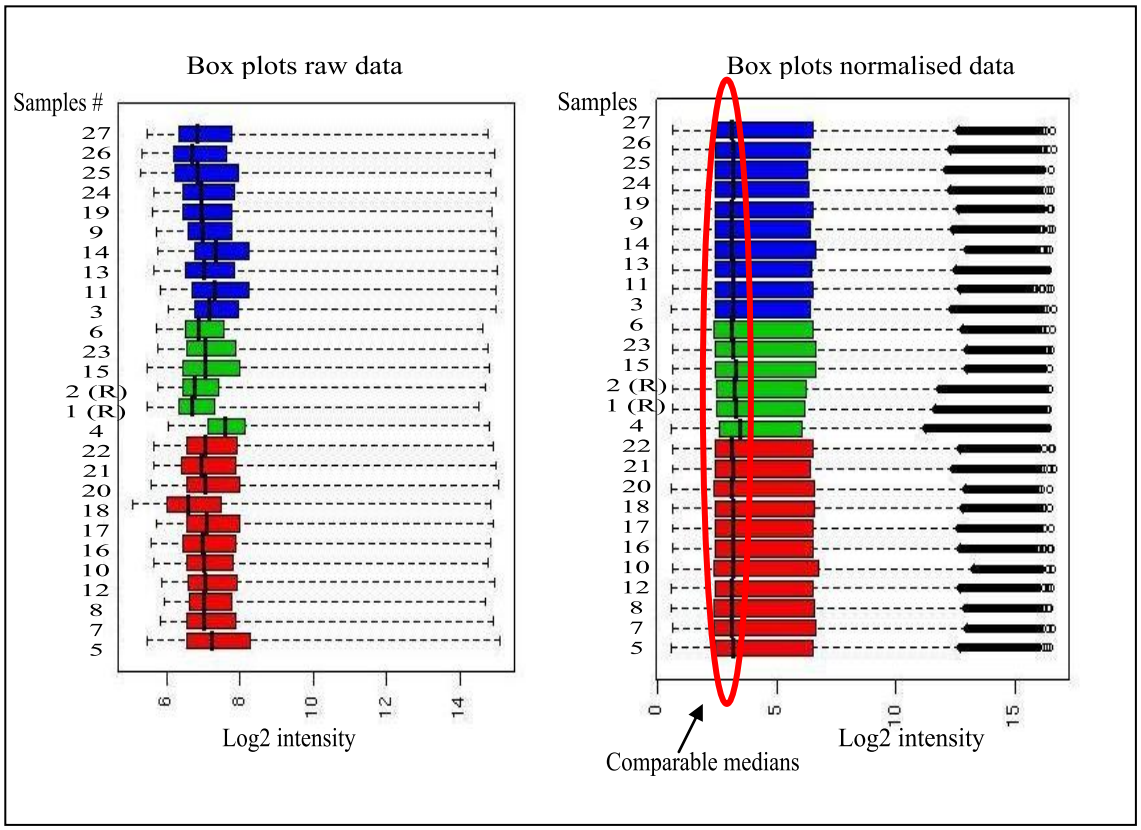
Chip	Ploy-A controls	3'/5' ratio GAPDH	ß-actin	Hybridisation controls	% gene present	Back ground	Raw noise	Scaling factor
	Dap 2306.55			*Cre* 6986.89				
14	*Lys* 248.00 *Phe* 221.90 *Thr* 521.30 *Dap* 1557.16	1.55	3.00	*BioB* 114.26 *BioC* 109.02 *BioD* 1428.08 *Cre* 5413.53	48.9	73.71	2.3	0.682
15	*Lys* 179.21 *Phe* 128.06 *Thr* 207.95 *Dap* 1068.76	**3.69**	**11.26**	*BioB* 272.41 *BioC* 289.29 *BioD* 2552.6 *Cre* 8671.71	52	55.18	1.94	0.778
16	*Lys* 338.84 *Phe* 336.87 *Thr* 588.47 *Dap* 1986.02	1.74	3.96	*BioB* 301.34 *BioC* 313.41 *BioD* 2986.91 *Cre* 9843.64	45.7	57.36	1.95	1.002
17	*Lys* 158.76 *Phe* 126.52 *Thr* 198.62 *Dap* 813.02	1.85	2.77	*BioB* 222.91 *BioC* 219.59 *BioD* 2542.9 *Cre* 8433.6	45.7	61.93	2.04	0.88
18	*Lys* 281.12 *Phe* 254.85 *Thr* 369.28 *Dap* 1590.98	1.44	2.69	*BioB* 265.03 *BioC* 273.4 *BioD* 3083.47 *Cre* 10460.94	44.4	43.19	1.44	1.017
19	*Lys* 166.48 *Phe* 162.72 *Thr* 291.31 *Dap* 1074.75	1.73	3.66	*BioB* 263.16 *BioC* 269.35 *BioD* 3098.78 *Cre* 10401.02	43.9	60.3	1.96	1.02
20	*Lys* 159.62 *Phe* 145.45 *Thr* 226.66 *Dap* 811.04	1.39	3.58	*BioB* 213.57 *BioC* 229.1 *BioD* 2625.79 *Cre* 8330.86	47	64.11	2.09	0.796
21	*Lys* 162.26 *Phe* 122.61 *Thr* 211.22 *Dap* 841.5	1.69	1.82	*BioB* 268.73 *BioC* 271.76 *BioD* 3085.56 *Cre* 10721.94	42.5	58.4	1.91	1.069
22	*Lys* 150.83 *Phe* 109.2 *Thr* 165.88 *Dap* 686.27	1.61	3.97	*BioB* 213.09 *BioC* 219.6 *BioD* 2430.91 *Cre* 8451.39	44.4	66.34	2.11	0.896
23	*Lys* 256.73 *Phe* 222.85 *Thr* 355.37 *Dap* 1382.18	2.99	5.88	*BioB* 236.05 *BioC* 239.48 *BioD* 2516.03 *Cre* 9094.16	47.1	64.3	2.07	0.993
24	*Lys* 240.5 *Phe* 162.62 *Thr* 297.28 *Dap* 1198.63	1.19	1.98	*BioB* 229.58 *BioC* 263.29 *BioD* 2899.7 *Cre* 10235.29	41.3	60.09	1.95	1.112
25	*Lys* 264.57 *Phe* 266.75 *Thr* 434.89 *Dap* 1433.26	1.24	2.19	*BioB* 184.76 *BioC* 182.74 *BioD* 2136.91 *Cre* 7445.37	40.7	50.61	1.74	0.960
26	*Lys* 145.33 *Phe* 143.4 *Thr* 247.73 *Dap* 806.72	1.43	1.57	*BioB* 231.31 *BioC* 233.83 *BioD* 2349.94 *Cre* 8784.93	43.4	49.68	1.67	1.070
27	*Lys* 191.45	1.39	2.26	*BioB* 187.69	44.2	56.68	1.85	1.061

Chip	Ploy-A controls	3'/5' ratio GAPDH ß-actin	Hybridisation controls	% gene present	Back ground	Raw noise	Scaling factor
	Phe 150.91 *Thr* 297.77 *Dap* 982.31		*BioC* 189.76 *BioD* 2268.69 *Cre* 8069.67				
GQ	Expressed genes	≤ 3	Increasing intensity from BioB to Cre	< 10%*	20-100	Similar values	≤ 2 SD

Samples were numbered from 1 to 27 starting with the 1st batch going through to the 4th batch samples. Both samples 1 and 2 were the technical replicates (R) and had comparable results. Samples 10 and 15 have failed the 3'/5' ratio of both genes. Samples 7, 8, 16, 19, 20, 22 and 23 have failed the 3'/5' ratio of the ß-actin gene only. The samples that have failed any criteria were marked in red. *Less than 10% difference between technical replicates. GQ: Good quality. SD: Standard deviation.

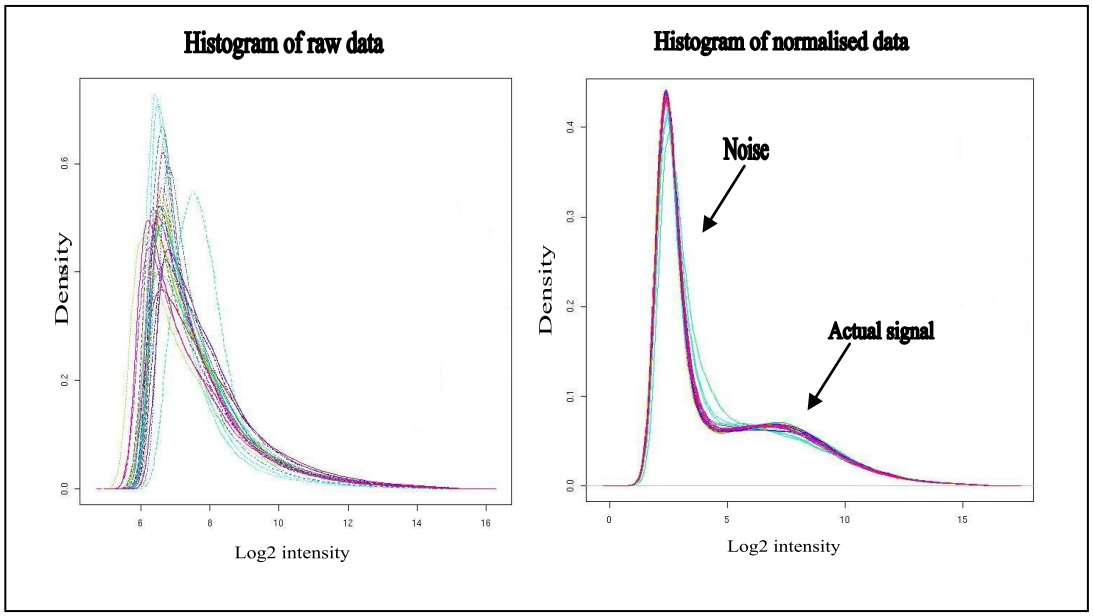
Pre-processing data and normalisation

Box plots



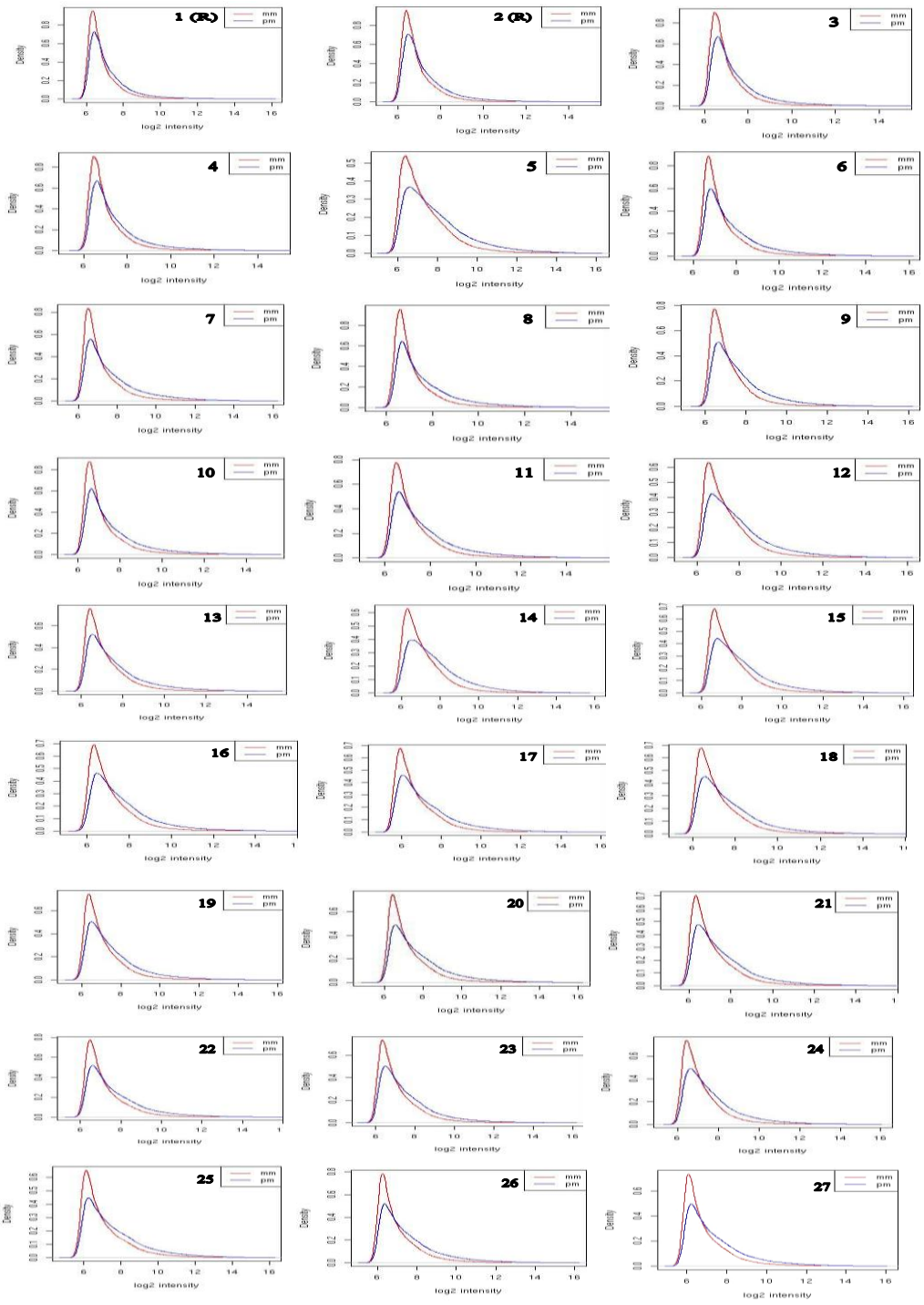
Box plots of both raw and normalised data. Samples were colour coded based on the horizontal facial deformity (blue, Class III; green, Class II; red, control patients). The black vertical line present in the middle of each box plot represents the median log intensity for each chip. In the raw data it is clear that the position of the median line is largely different between samples as it is affected by the raw noise and un-specific hybridisation. Once the data have been normalised and all systematic variations have been removed, the median line of all samples was aligned close to teach other and the data were more comparable.

Perfect-match (PM) signal histograms



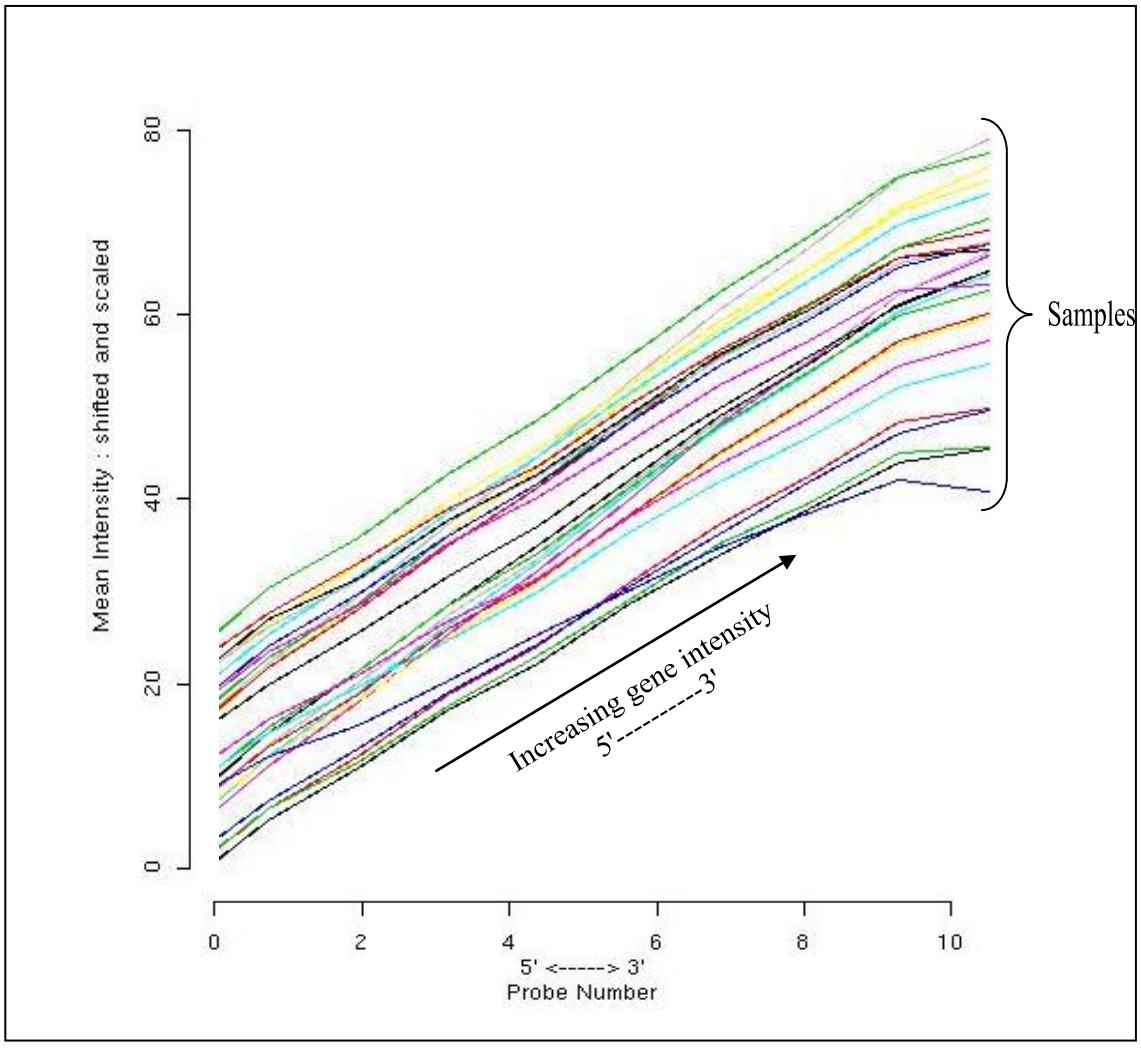
This histogram is a measurement of the density of the probe intensities of both the PM probes and the noise. Typical histogram appearance of normalised PM signals which is separated from the noise would appear with the density skewed to the right with 2 distinct peaks. One is long and narrow that represents the noise and the other one is small and broad that is the actual PM signal. All samples showed typical PM histogram appearance with no obvious outliers.

Histogram of PM vs. MM



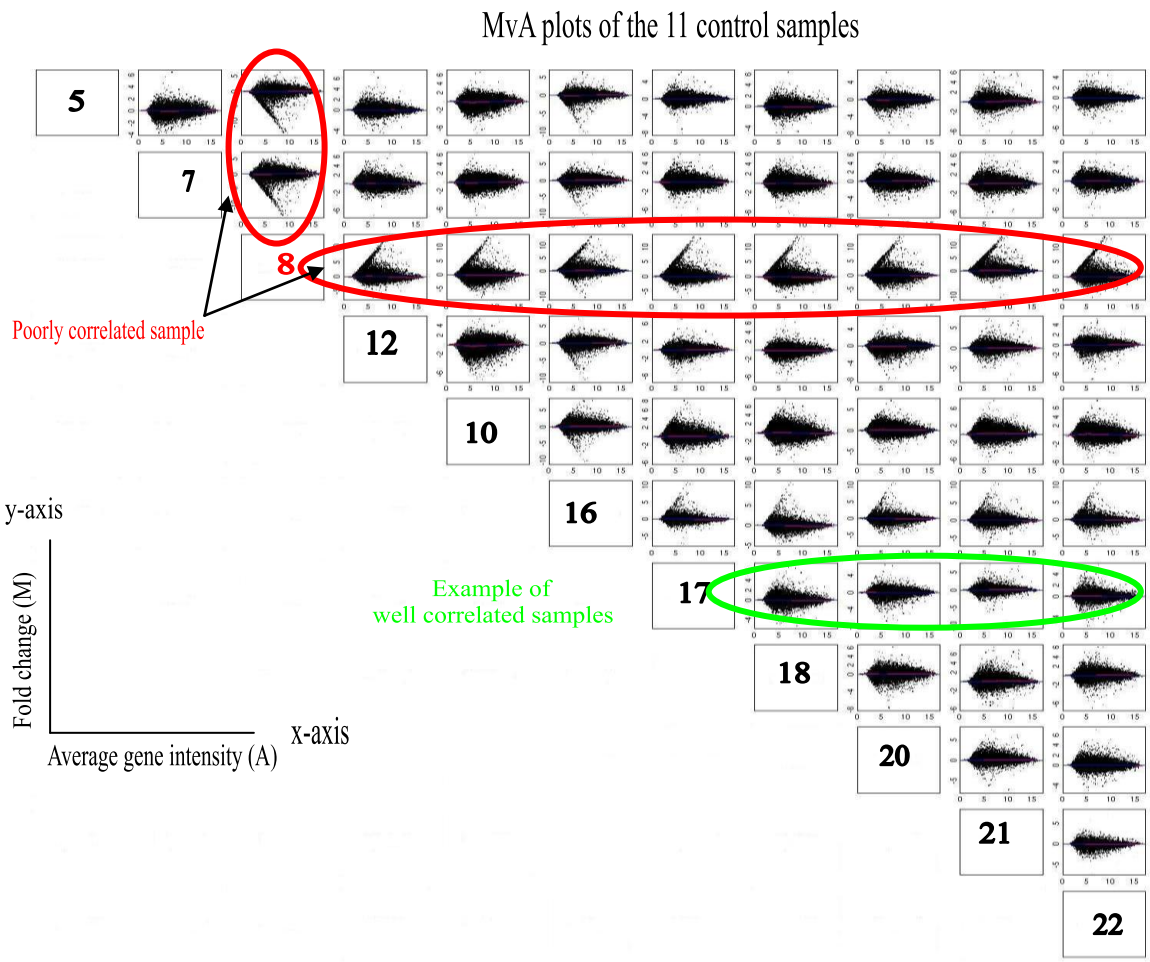
The mis-match (MM) probes measure non-specific hybridisation while PM probes measure specific hybridisation. It is therefore, expected that the PM probes will produce strong intensities while the MM probes weaker intensities. All 27 samples had a typical PM-MM histogram appearance with the MM probes (red colour peak) having a narrower intensity peak than the PM probes (blue colour peak).

RNA degradation plot



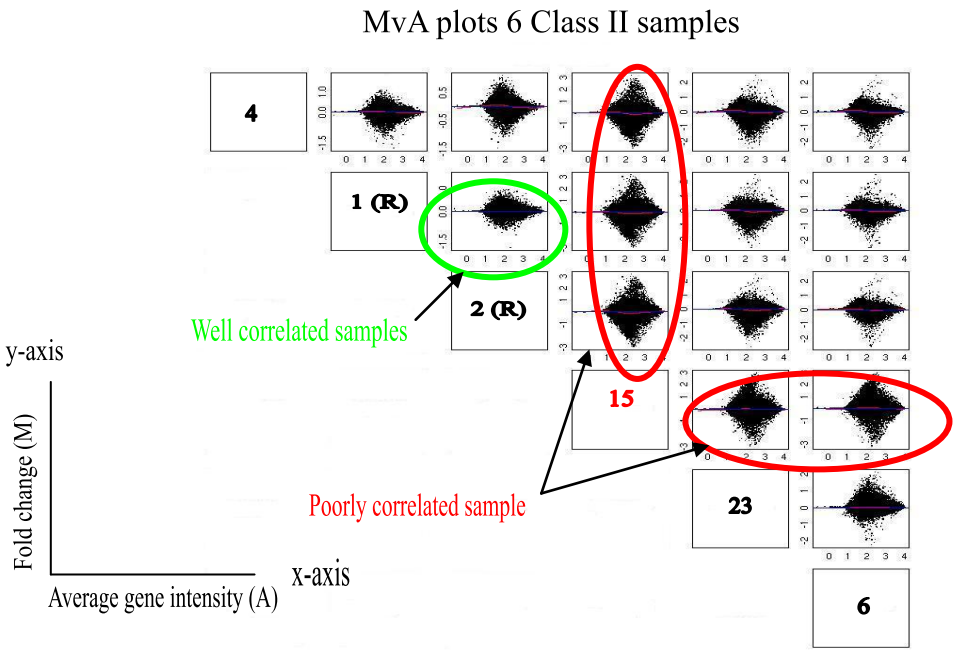
The 0 probe presenting the most 5' sequence and the 10th probe is the most 3' sequence. Since RNA degradation starts at the 5' end, and labelling occurs from the '3 end, therefore, a typical RNA degradation plot would show increasing intensities from the 5' to the 3' end. All 27 samples had increasing intensities from the 5' probes to the 3' probes.

MvA plots of the controls



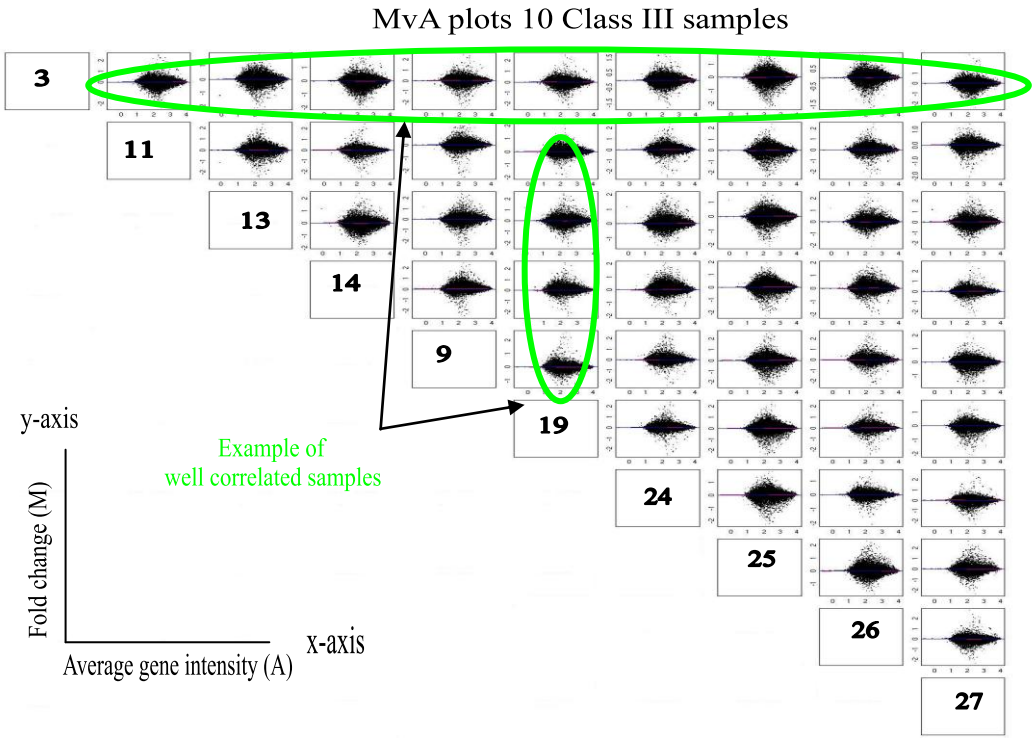
Each MvA plot was a graphical representation of two samples only, where the fold-change (M) at the y-axis is plotted against the average fluorescence intensity (A) of both samples at the x-axis. If both samples were highly comparable they will have a symmetric appearance around the x-axis (leaf like appearance) similar to the correlation of sample 17 with samples 18, 20, 21 and 22 (marked in green). While if a sample was not correlated to the other samples, the leaf like appearance will have extensions, similar to sample 8 in relation to all other samples (marked in red).

MvA plots of the Class II group



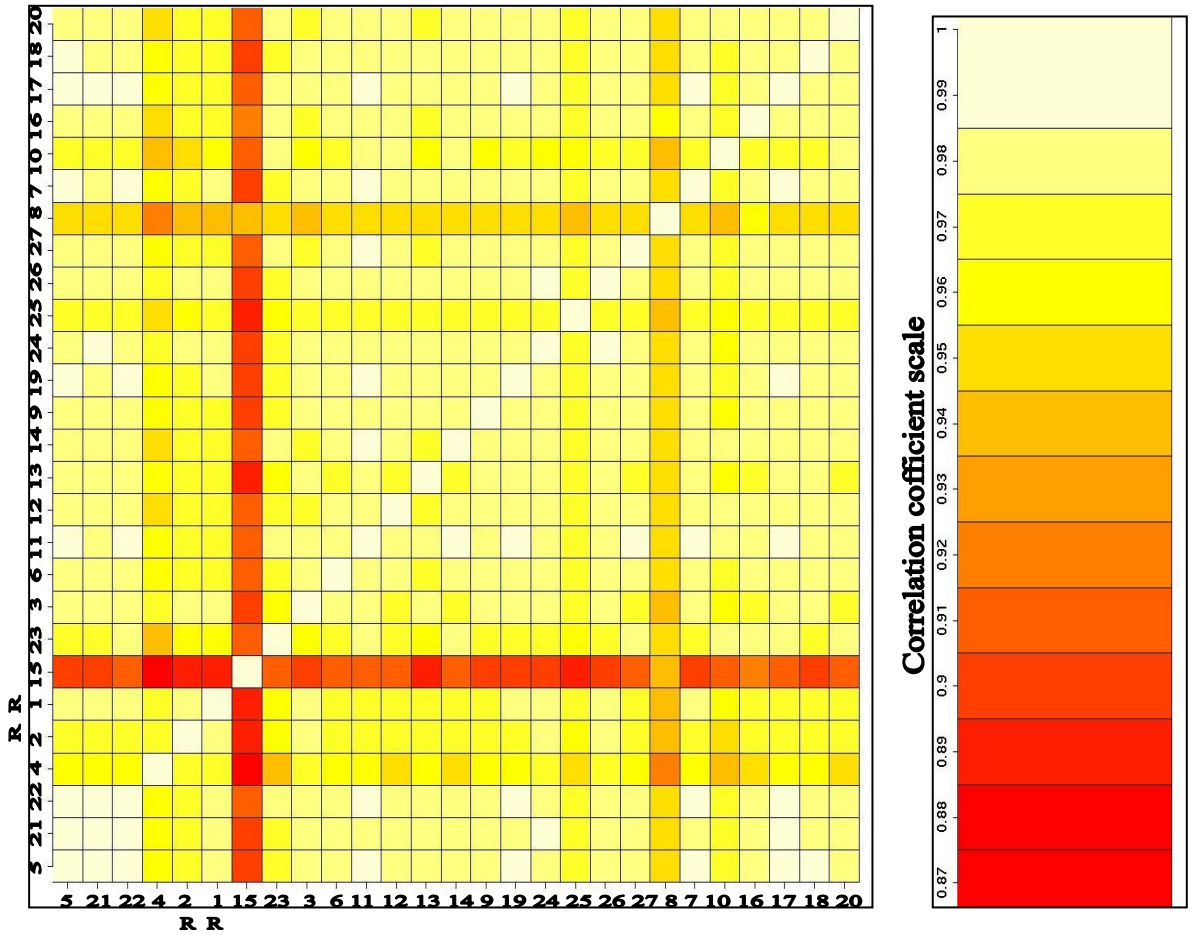
Sample 15 shows vertical widening of the leaf like appearance which indicates a large fold change variation and poor correlation to all other samples (marked in red). Both technical replicates were well correlated (marked in green).

MvA plots of the Class III group



All chips within this group were correlated where all samples showed a leaf like appearance with no extensions and no marked increase in the fold change. Samples 3 and 19 (marked in green) are examples of good MvA correlations.

Correlation plot



The heatmap was a chip-chip Spearman's rank correlation coefficient that is colour coded. The lighter the colour, the greater the correlation. Both technical replicates (R) were highly correlated (0.98). Both samples 8 and 15 are showing darker colours compared to other samples, with sample 15 being less correlated to other samples than chip 8.

Summary of the study following MIAME guidelines

MIAME guidelines	Description
Study design *Main aim*	-Ascertain masseter muscle candidate genes in relation to craniofacial deformities
Study type	-Case control study (deformity vs. control)
Experimental factor	-Gene expression variation
Experimental design	-Long face vs. control (vertical classification) -Class II vs. Class III vs. control (horizontal classification)
Quality control	-Biological replicates included 11 control and 18 deformity patients -Technical replicates included 2
Sample description *Sample origin*	-Fresh human masseter muscle biopsies collected prior to any orthodontic intervention during the surgical removal of third molars
Sample manipulation	-Samples stored in tubes containing RNA*later*® reagent at -80 C° until RNA extraction
RNA extraction	-Optimised protocol using the lysing matrix D®, the FastPrep® machine, 20 seconds, speed 6, 5 minutes cool down on ice, repeated twice. Qiagen™ RNeasy® mini kit protocol with DNase digestion step for 15 minutes.
RNA quality control	-Good quality shown by the Bioanalyser profile Nano LabChip® kit
Microarray laboratory design *Amplification for microarray*	-Two cycle amplification protocol
Labelling	-Labelled cRNA (standard Affymetrix® protocol)
Hybridisation	-Standard Affymetrix® protocol
Spike controls	-Standard Affymetrix® protocol
Data analysis *Data extraction*	-Standard Affymetrix® procedure
Raw data	- CHP file generated using the Affymetrix® suite -MAS 5.0
Pre-processing data	-Standard Affymetrix® procedure
Normalised data	-GCRMA normalisation
Results	-Long face vs. controls (12 up- and 19 down-regulated in long face) -Class II vs. control (3 up- and 9 down-regulated in Class II) -Class III vs. control (15 up- and 21 down-regulated in Class III) -Class II vs. class III (5 up- and 7 down-regulated in Class II) -All deformity vs. control (6 up- and 19 down-regulated)
Array design *Array name*	HG U133 Plus 2.0 Array GeneChip®
MIAME: Minimum Information About a Microarray Experiment	

Appendix E. Publications

POSTER

A.M Ödman, N.P Hunt, S. Kiliaridis, **H.A. Matloub Moawad** and M.P. Lewis. Do Msticatory functional changes influence master muscle of adult rats?. Presented at the PEF IADR conference, Sep 10-12, 2008, London, UK.