Matching genotype to phenotype in a detailed assessment of lip morphology

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Abstract

Background: Craniofacial morphology has been reported to be highly heritable, but prior to this research, little was known as to which genetic variants influence normal lip phenotypes. Much of the previous genetic research involved assigning rare genetic mutations to craniofacial abnormalities, giving little insight as to what causes normal variation. More recent studies have attempted to assign genetic variants to facial phenotypes using landmarking methods, however, these markers are sparse in the lip region. Considerable variation of lip morphology exists, which is not amenable to landmarking methods.

The aim of this study was to investigate the biological basis of lip phenotypes.
Objectives

- Develop a robust, reproducible classification system of lip phenotypes
- Measure the prevalence of lip phenotypes within a population sample of 4,747
- Assess for associations of lip phenotypes with other lip phenotypes
- Assess if there were any sex variations of lip phenotypes
- Assess the reproducibility of the classification system with other acquisition methods
- Perform a genome-wide association study (GWAS) of lip phenotypes
- Assess for associations of non-syndromic cleft lip/palate (NSCL/P) SNPs to lip phenotypes
- Perform a case/control study to assess the prevalence of lip phenotypes amongst unaffected biological parents of cleft and non-cleft children
- Assess the predictive capability of lip phenotypes as a precursor of cleft risk

Methods: Three-dimensional laser scanned facial images were obtained of 4,747 subjects recruited from the Avon Longitudinal Study of Parents and Children (ALSPAC). A total of 102 individuals were recruited from the University Dental Hospital, Cardiff to assess the reproducibility of the classification system with other acquisition methods. Genetic data was available for 3,687 ALSPAC subjects for the discovery phase of GWAS, and 3,215 digital photographs of Australian twins for the replication phase. Images of 597 3dMD case-parent trios and controls were obtained through FaceBase, to assess the prevalence of lip phenotypes amongst unaffected biological parents of cleft and non-cleft children.

Results: Twenty-five reproducible lip phenotypes have been described. Most phenotypes occur in combinations, except mentolabial fold and chin dimple, which are distinct. Prevalence of features and sex dimorphism are also explained. A modification of the lip scale is required for facial shells with reduced surface resolution taken with 3dMD or digital photographs.

A discovery GWAS of 3,687 ALSPAC subjects revealed two new GWAS significant hits: chin dimple and *DOCK1* ($P=2x10^{-8}$) and mentolabial fold and *CDH4* ($P=1x10^{-8}$). Both genes have been reported to play a role in tumour pathways (Du, C. *et al*, 2011, Laurin, M. *et al*, 2013), and *CDH4* may have a role to play in muscle development (Nogueira, J.M. *et al*, 2015). In addition, 29 near-hit associations were found with 18 lip phenotypes ($P<10^{-7}$). Replication was attempted for 2 lip phenotypes (chin dimple and mentolabial fold). However, it was not possible to replicate these findings.

Genotype analysis discovered associations with 14 out of 17 candidate NSCL/P SNPs with 21 out of 25 lip phenotypes. The main findings were the association of *NOG* and skeletal II pattern ($P=1.58x10^{-6}$) and V-shaped Cupid's bow ($P=2.48x10^{-3}$) with 8q24. A generated NSCL/P genetic allele score demonstrated association with V-shaped

Cupid's bow ($P=2x10^{-4}$), narrow philtrum ($P=3x10^{-4}$), upturned commissures ($P=0.03$) and deep philtrum ($P=0.045$). The prevalence of 12 out of 23 lip phenotypes was found to be higher amongst case-parent trios compared with control parents. Case mothers had increased prevalence of convex upper lip contours, upper lip groove, absent lower lip groove, flat lower lip contour and angular lower lip tone with bumping. Case fathers had absent lower lip vermilion borders and double borders.

Conclusions: A robust and reliable scale has been presented, which allows categorisation of lip phenotypes. Considerable variation exists within the ALSPAC population of 15 year olds, including some rare phenotypes and evidence of sex dimorphism. Lip phenotypes tend to appear in combinations with other lip phenotypes, whilst chin dimple and mentolabial fold are generally distinct.

Discovery GWAS indicated genotype/phenotype associations with chin dimple and *DOCK1* ($P=2x10^{-8}$) and mentolabial fold and *CDH4* ($P=1x10^{-8}$). However, this was not replicated in an independent sample.

NSCL/P SNPs and combined high-NSCL/P genetic alleles affect lip phenotypes, and appear to induce a narrow philtrum, V-shaped Cupid's bow and a skeletal II pattern.

Parents of cleft children had higher prevalence of some lip phenotypes compared with control parents. As such, this study proposes that certain lip phenotypes may be utilised as subphenotypic markers of cleft risk.

Contents

List of Tables

List of Figures

List of abbreviations

2D	Two-dimensional
3D	Three-dimensional
3dMD	3dMD face dynamic system (3dMD Inc., Atlanta, GA, USA)
ALSPAC	Avon Longitudinal Study of Parents and Children
BRIM	Bootstrapped response-based imputation modelling
CBCT	Cone Beam Computerised Tomography
CL	Cleft Lip only
CLP	Cleft Lip and Palate
CL/P	Cleft Lip and/or Palate
CPO	Cleft Palate only
CT	Computerised Tomography
FAS	Foetal Alcohol Syndrome
GWAS	Genome Wide Association Study
MRI	Magnetic resonance imaging
NSCL/P	Non-syndromic Cleft Lip and/or Palate
OFC	Orofacial clefts
PC	Principal Component
PCA	Principal Component Analysis
SCL/P	Syndromic Cleft Lip and/or Palate
SNP	Single Nucleotide Polymorphs

Introduction

The lips are important for verbal and non-verbal communication, mastication, maintaining an oral seal and are seen as an important factor in facial and sexual attractiveness. A significant amount of literature has been written on the lips in respect to cleft lip and palate (CLP) repair, (Veau, V., 1931, Millard, D.R., 1958), change in lip contour as a result of conventional orthodontic treatment (Bittner, C. *et al*, 1990) or surgical positioning of the maxilla and mandible (Dann, J.J., 3rd *et al*, 1976, Ferrario, V.F. *et al*, 1999).

Evaluating lip morphology

Traditionally in the clinical setting, lip morphology has been limited to simple parameters such as, lip length (Mamandras, A.H., 1984, 1988), lip competence (or lack of) and lip thickness (Nanda, R.S. *et al*, 1990). The nasolabial angle is also of interest, and accounts for the angle of intersection between the columella of the nose and the inclination of the upper lip and has average values (Farkas, L.G. *et al*, 1984, Farkas, L.G., 1994). Orthodontists perform an assessment of the lips as part of the initial assessment of the face prior to treatment planning. Orthodontic movement of the teeth alone, or in association with a surgical procedure, will affect the soft tissues, and consequently facial appearance (Dann, J.J., 3rd *et al*, 1976, Ferrario, V.F. *et al*, 1999, Wermker, K. *et al*, 2014).

The majority of previous research in this area has involved anthropometric measurements of multiple facial landmarks (Farkas, L.G., 1994), geometric morphometrics (Claes, P. *et al*, 2014) and principal component analysis (PCA) (Toma, A.M. *et al*, 2012). Few studies have involved an assessment of characteristric features (Mori, A. *et al*, 2005, Carey, J.C. *et al*, 2009, Hennekam, R.C. *et al*, 2009). Therefore, there is sparse population-based data, which identifies and describes normal lip phenotypes.

Genotype-phenotype associations

Facial development is influenced by genetic (Grosen, D. *et al*, 2010a, Peng, S. *et al*, 2013) and environmental factors (Abel, E.L., 1995, Honein, M.A. *et al*, 2007, Al Ali, A. *et al*, 2014), and animal models, have demonstrated that craniofacial development is tightly regulated by chemical mediators (Francis-West, P.H. *et al*, 2003). Much of the

research encompassing genetics of the lip region has previously involved cleft susceptibility (Cobourne, M.T., 2004), however, little is known as to how these mediators affect normal facial variation.

Genotype-phenotype associations of the lip region could be assessed using genome-wide association study design, which have been utilised in assigning genetic association to common population variations (Bush, W.S. *et al*, 2012, Paternoster, L. *et al*, 2012, Liu, F. *et al*, 2012c).

Research aims and objectives

The overarching aim of this study was to investigate the biological basis of lip phenotypes

The objectives were to:

- Develop a robust, reproducible classification system of lip phenotypes
- Measure the prevalence of lip phenotypes within a population sample of 4,747
- Assess for associations of lip phenotypes with other lip phenotypes
- Assess if there were any sex variations of lip phenotypes
- Assess the reproducibility of the classification system with other acquisition methods
- Perform a genome-wide association study (GWAS) of lip phenotypes
- Assess for associations of non-syndromic cleft lip/palate (NSCL/P) single nucleotide polymorphs (SNPs) to lip phenotypes
- Perform a case/control study to assess the prevalence of lip phenotypes amongst unaffected biological parents of cleft and non-cleft children
- Assess the predictive capability of lip phenotypes as a precursor of cleft risk

Contributions

This thesis presents a novel method of assessing lip shape in a detailed, systematic approach. The main contributions were derived by:

- Assessing both the intra and inter examiner reproducibility of a scale that categorises lip phenotypes.
- Determining prevalence and associations of lip morphological features in a large population of 15-year-old Caucasians.
- Determining reproducibility of lip phenotype categories with a different acquisition technique and comparing colour and greyscale facial surface images.
- Identifying two statistically significant associations, and twenty-two close hits.
- Identifying the effect of common variant NSCL/P SNP's on lip morphology.
- Determining differing lip morphology between unaffected parents of cleft children and control parents in a case/control study.

Organisation of thesis

Chapters 1-3: Literature review and aims and objectives

Chapter 1 describes anatomy of the lip region, fundamental concepts of existing methods of analysing lip phenotypes and various acquisition methods. Chapter 2 investigates various theories of normal lip development and clinical conditions arising when lip development is disrupted. It also explores the concepts of genome-wide association study (GWAS) and examines the current understanding of the genes involve in CL/P. Chapter 3 then goes on to list the aims and objectives of this study.

Chapter 4-8: Experimental work, results, discussions and conclusions

Chapter 4 addresses the methodology used to develop the classification tool used in this study and describes the experimental results, including reproducibility, prevalence of features and sex dimorphism. Chapter 5 explores the validity of using the classification scale (Figure 4.4) for images captured using 3dMD face dynamic system (3dMD Inc., Atlanta, GA, USA) and offers a modified classification scale. Chapter 6 describes genotype/phenotype association results using GWAS. Chapter 7 presents the results of the candidate gene association study (NSCL/P SNPs), and the effect of common variants on lip phenotypes. Chapter 8 describes the case-control study, assessing the prevalence of lip phenotypes amongst unaffected parents of NSCL/P children, versus controls.

Chapter 9: Overall Summary

Chapter 9 gathers all the main findings of the study.

Chapter 10: Future Studies

Chapter 10 displays recommendations for future research.

Publications from thesis

- Wilson C, Playle R, Toma A, Zhurov A, Ness A, Richmond S. The prevalence of lip vermilion morphological traits in a 15-year-old population. Am J Med Genet A. 2013 Jan; 161A (1): 4-12
- S. Richmond, A.M. Al Ali, L. Beldie, Y.T. Chong, A. Cronin, J. Djordjevic, N.A. Drage, D.M. Evans, D. Jones, Y. Lu, D. Marshall, J. Middleton, G. Parker, L. Paternoster, R.A. Playle, H. Popat, P.L. Rosin, K. Sidorov, A.M. Toma, B.Walker, C.Wilson, A.I. Zhurov. Detailing patient specific modeling to aid clinical decision-making. Edition: 1st, Publisher: Springer, Editor: Begoña Calvo Lopez, Estefanía Peña, ISBN: 978-94-007-4552-0

Chapter 1: Lip anatomy and phenotyping

1.1 Introduction

In the orthodontic clinical setting, lips have been traditionally assessed according to competence, length (Mamandras, A.H., 1984, 1988, Peck, S. *et al*, 1992) and the nasolabial angle (Farkas, 1992b).

Numerous articles have been published in relation to growth changes, recording average lip length, width and fullness (Farkas, L.G. *et al*, 1984), or changes in lip prominence as a result of conventional orthodontic treatment or surgical positioning of the maxilla and mandible (Dann, J.J., 3rd *et al*, 1976, Ferrario, V.F. *et al*, 1999).

Traditional methods of assessing the lips have involved recording facial landmarks, and subsequent analysis using measurements or geometric morphometrics (Bookstein, F.L., 1997), a few studies have also defined lip variation as a result of characteristic features (Mori, A. *et al*, 2005, Carey, J.C. *et al*, 2009, Hennekam, R.C. *et al*, 2009).

This chapter explores the anatomy of the lips and their surrounding region; it also considers methods of assessment and capturing, as well as various methods of phenotyping.

1.2 Anatomy of the lips

The lip region comprises the base of the nose (subnasale) to the tip of the chin (gnathion) vertically, and between the commissures laterally, incorporating the philtrum and cupids bow. There are well-documented, reproducible landmarks that describe the features of the lip region (Figure and Table 1.1).

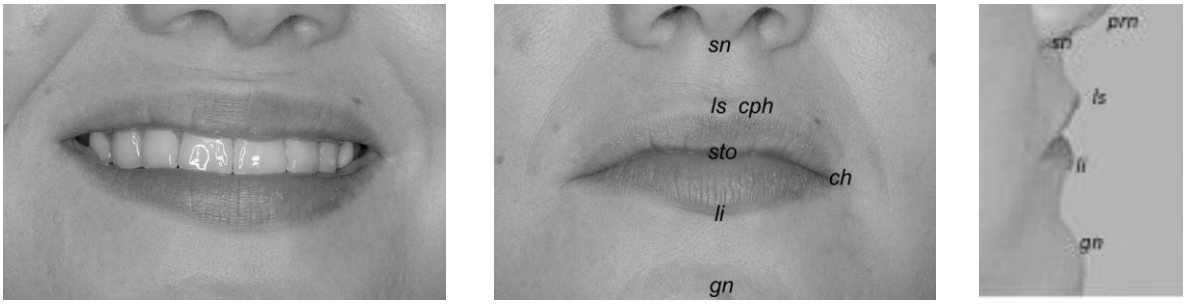


Figure 1.1 Landmarks and features of the lips
The lips are assessed with the subject relaxed (centre and right), as they distort with facial movement (left)

Table 1.1 Definitions of landmarks and features of the lips	
Landmarks	**Definitions**
Subnasale (sn)	Point at which the nasal septum and upper lip meet (Farkas, L.G. *et al*, 1984)
Labiale superius (ls)	Mid-point of the upper vermilion line (Farkas, L.G. *et al*, 1984)
Crista philtre (cph)	Point on the elevated margins of the philtrum just about the vermilion line (Farkas, L.G. *et al*, 1984)
Stomion (sto)	The labial fissure; slit-like space between the lips (Carey, J.C. *et al*, 2009). Opening of the oral cavity (Farkas, L.G. *et al*, 1984)
Cheilion (ch)	Point located at the angle (corner) of the oral cavity (Farkas, L.G. *et al*, 1984)
Labiale inferius (li)	Mid-point of the lower vermilion line (Farkas, L.G. *et al*, 1984)
Gnathion (gn)	The midpoint of the most anterior, prominent, bony part of the mandible (Mosby, 2009)
Features	
Philtrum (sn-ls)	The philtrum is the area of infranasal depression that runs as a vertical groove from the base of the nose (sn) to the vermilion border (ls). It is bordered by two lateral ridges, sometimes called pillars (Hennekam, R.C. *et al*, 2009)
Cupid's Bow (cph–ls–cph)	The Cupid's bow is the contour of the line formed by the vermilion border of the upper lip. In the frontal view, this line resembles an archer's bow, which curves medially and superiorly; running from the commissures to the paramedian peaks located at the base of the pillars of the philtrum (cph) with an inferior convexity lying between those peaks (ls). There are two variants; absent and exaggerated (Carey, J.C. *et al*, 2009)
Nasolabial angle (prn–sn–ls)	The angle formed by the labial surface of the upper lip at the midline (sn-ls) and the inferior border of the nose (sn-prn). It is a measure of the relative protrusion of the upper lip (Mosby, 2009)
Vermilion	The vermilion is the red part of the lips. It is covered with a specialised stratified squamous epithelium, which is in continuity with the oral mucosa (Carey, J.C. *et al*, 2009) The lips vary in shape with facial movement (Figure 1.1); therefore for consistency the lips must be assessed with the subject relaxed, and in natural head position (Solow, B. *et al*, 1971)
Vermilion border	The rim of paler skin that demarcates the vermilion from the surrounding skin (Carey, J.C. *et al*, 2009)
Commissures (ch-ch)	The oral commissures form where the lateral aspects of the vermilion of the upper and lower lips join. The cheilion (ch) is the anthropological landmark located at this site (Carey, J.C. *et al*, 2009)
Skeletal pattern	Assessment of jaw discrepancy between the maxilla and mandible in relation to the cranial base (Roberts-Harry, D. *et al*, 2003).

1.2.1 Underlying anatomical features affecting lip shape

The lips surround the oral cavity, and their position is affected by the prominence of the anterior teeth. The upper incisors provide support to the upper lip, and their inclination can affect the relative protrusion and perceived fullness of the lips. The inclination of the lower incisors also affects the degree of indentation of the lower lip. The position, tonicity and thickness of the lips can also affect the position of the teeth. A high lower lip line in a skeletal II pattern, may affect the inclination of the upper incisors, depending on whether the lower lip sits behind the upper incisors or in front of the incisors (Figure 1.2).

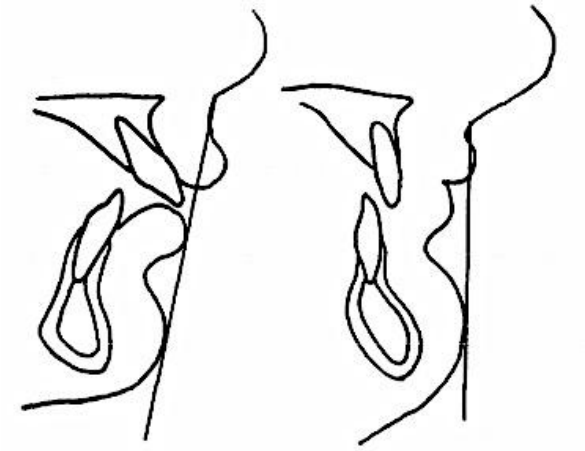


Figure 1.2 Variation of horizontal lip posture and its effect on the position of the incisors
In certain skeletal facial patterns, the inclination of the upper incisors are affected by the position of the lower lip; leading to proclined upper incisors when the lower lip lies behind the upper incisors (left) or retroclined upper incisors if the lip lies in front of the incisors (right) (Burstone, C.J., 1967, Mcintyre, G.T. *et al*, 2006)

The fullness and thickness of lips vary with age, with periods of increase in both dimensions during maturity up to the age of 16-18years. After the early twenties, there is subsequent flattening and retrusion (Bishara, S.E. *et al*, 1998). The lips are also influenced by ethnicity (Lew, K.K. *et al*, 1993, Wilkinson, C.M. *et al*, 2003, Zhu, L.Y. *et al*, 2008, Seager, D.C. *et al*, 2009, Wong, W.W. *et al*, 2010), with Caucasians having generally thinner and less full lips compared to African Americans (Astley, S.J. *et al*, 1995).

1.2.2 Musculature

The musculature underlying the lip region originates from the first and second pharyngeal arches, and constitutes the group of muscles collectively known as the muscles of mastication and facial expression, respectively.

The musculature surrounding the oral cavity (Figure 1.3) is the orbicularis oris muscle, which acts to open, pucker or compress the lips. Observations of serial histological sections illustrate that the orbicularis oris muscle consists of two distinct layers; a deep and a superficial layer (Latham, R.A. *et al*, 1976). However, it is not a simple sphincter muscle, as other muscle fibres that insert into it, influence it.

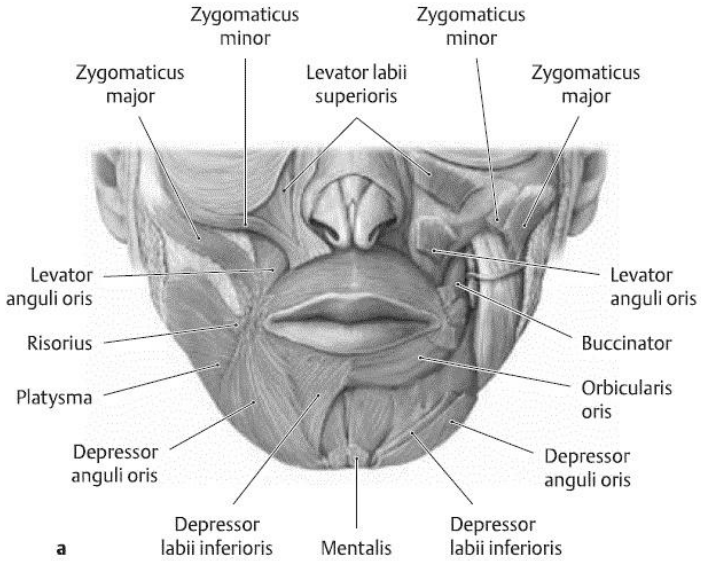


Figure 1.3 Diagram displaying the peri-oral muscles (Caldwell, T., 2014)

Most of the deep layer fibres originate from the buccinator muscle, which pass across the lips from side to side, whereas some of the middle fibres cross each other at the commissures. The superficial layers consist of fibres from the levator anguli oris (caninus) and depressor anguli oris (triangularis) muscles and insert into the skin near the median line. The depressor anguli oris (triangularis) muscle fibres insert in the skin at the philtral ridges, and the superior bundle attaches to the anterior nasal spine. A few other muscle fibres integrate with the orbicularis oris muscle, they include the zygomaticus muscles, levator labii superioris and the depressor labii inferioris, these mainly act in an oblique direction.

Either side of the midline of the upper lip, there are two bands; the incisivus labii superioris laterally (arising from the alveolar border of the maxilla, opposite the lateral incisors and arching forwards) and the nasolabialis muscle medially, which connects

the upper lip to the nasal septum. It is the interval between the two medial bands, which corresponds with the depression of the philtrum (Latham, R.A. *et al*, 1976).

1.2.2.1 Vermilion border

The marginal part of the orbicularis oris muscle curls upon itself forming the pars marginalis (PM) anteriorly and the pars peripheralis (PP) posteriorly (Figure 1.4). Dissections of the upper lip have identified variations in the breadth and thickness of the pars marginalis in relation to the junction between the skin and the red vermilion at different sites of the upper lip. Towards the midline it is 2.5 +/-0.8mm above the vermilion border, and 3.1+/-0.4mm below, whilst at the commissures it is reduced to 1.6+/-0.4mm above, and 2.5+/-0.7mm below.

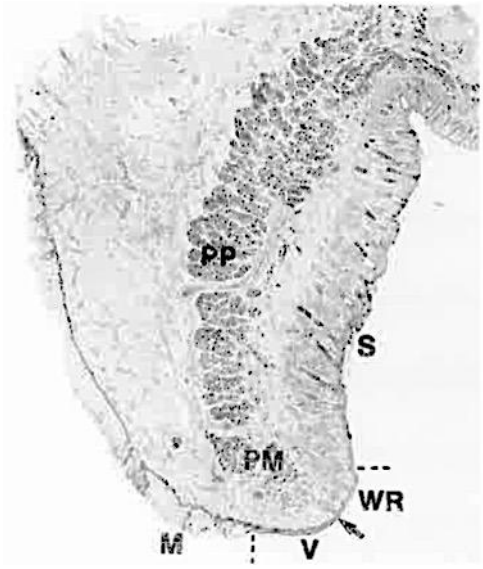


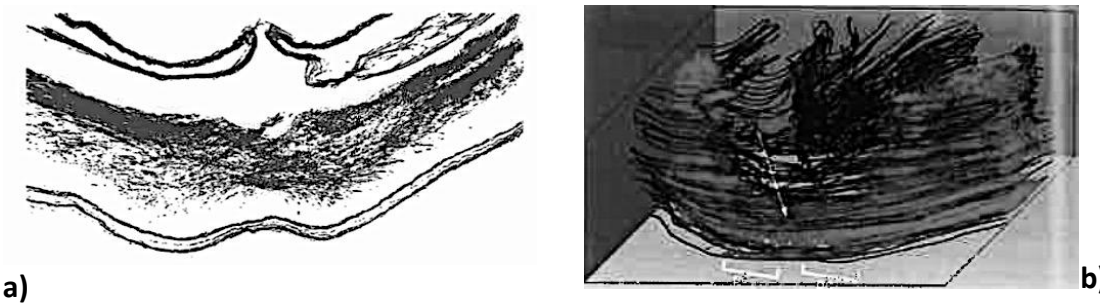
Figure 1.4 Histological section of a normal neonatal lip (Mulliken, J.B. *et al*, 1993)
(S - skin, WR - white roll, V - vermilion, M - mucosa, PM - pars marginalis, PP – pars peripheralis of the orbicularis oris muscle)

The thickness also varies, with the thickest part at the midline (2.0+/-0.5mm) and reducing (to 1.1+/-0.3) at the commissures (Hwang, K. *et al*, 2007). It could therefore be hypothesised that the pars marginalis contributes to the appearance of the rolled margin that comprises the vermilion border, and double border (Chapter 4) of the lips.

1.2.2.2 Philtrum

The philtrum is a feature of the lip at rest, as its shape starts to fade during facial expression. There are two schools of thought as to how the philtral ridges arise. The first theory proposes that the ridges are the result of lateral insertions of the muscles into the skin, and the groove therefore corresponds to the more compact decussation

zone at the midline with a relative absence of muscle fibre insertions (Latham, R.A. *et al*, 1976). Dissected cadavers indicated that fibres of the orbicularis oris muscle enter the lip on one side and cross over the midline to insert into the skin on the opposite side, lateral to the philtral groove. The philtral ridges appear to represent the mesial borders of bilateral muscle insertion zones of the lip, in which the fibres raise the level of the skin by splaying out and inserting into it. (Latham, R.A. *et al*, 1976). The second theory suggests that the ridges are formed by thickened dermis, dermal appendages and muscle fibres that decussate at the vermilion, this is supported by the fact that the philtrum usually appears normal on the contralateral side of a cleft in patients with unilateral CL (Namnoum, J.D. *et al*, 1997) (Figure 1.5).



a) **b)**

Figure 1.5 Diagrammatic representation of muscle fibres that decussate throughout the upper lip.
Muscle fibres decussate at the philtrum (left) (Latham, R.A. *et al*, 1976) and throughout the vermilion (right) (Namnoum, J.D. *et al*, 1997)

The levator labii superioris and nasialis muscles also contribute to philtral form. The levator labii superioris descends as far medially as the philtral ridge, and inserts into the vermilion border lateral to the mesial groove. The nasialis muscle inserts into the philtral ridges superiorly.

1.2.2.3 Cupid's bow

Gross and microscopic examination of the anterior projection of the pars marginalis supports the theory that the levator labii superioris muscle merges with the rim of orbicularis oris and inserts into the vermilion border, forming a labial arch and peak (Mulliken, J.B. *et al*, 1993). Thus, it is the action of the levator labii superioris lateral to the median groove, in conjunction with a depressor action by the orbicularis oris muscle on the medial tubercle that gives rise to the displacement of the vermilion border and the shape of the Cupid's bow.

It therefore seems plausible to suggest that it is the length or vigour of this muscle that determines the shape of the Cupid's bow. Therefore, one would expect an individual

with relative muscular weakness to have a flattened Cupid's bow appearance, compared to an individual without muscle weakness. In addition, as the tonicity of muscles reduces with ageing, it could also be suggested that the shape of the Cupid's bow would also modify with ageing, to a relatively flatter shape.

1.2.3 Blood Supply

The facial artery (branch of the external carotid artery) supplies the lips. The superior branch supplies the upper lip; its typical anatomy runs along the length of the vermilion border from the commissures and deep to the orbicularis oris muscle (Figure 1.6). In 23% of cases, this artery has been found to be unilateral (predominantly originating on the right hand side), crossing the midline to supply the whole lip (Tansatit, T. *et al*, 2014). The philtral arteries (the ascending and central) arise from the superior branch; the ascending artery coincides with the philtral column, and is the site where OFC occurs. An alteration in the anatomy of this artery may have a part to play in the aetiology of CL. The lower lip is supplied by the inferior branch, which runs along the chin.

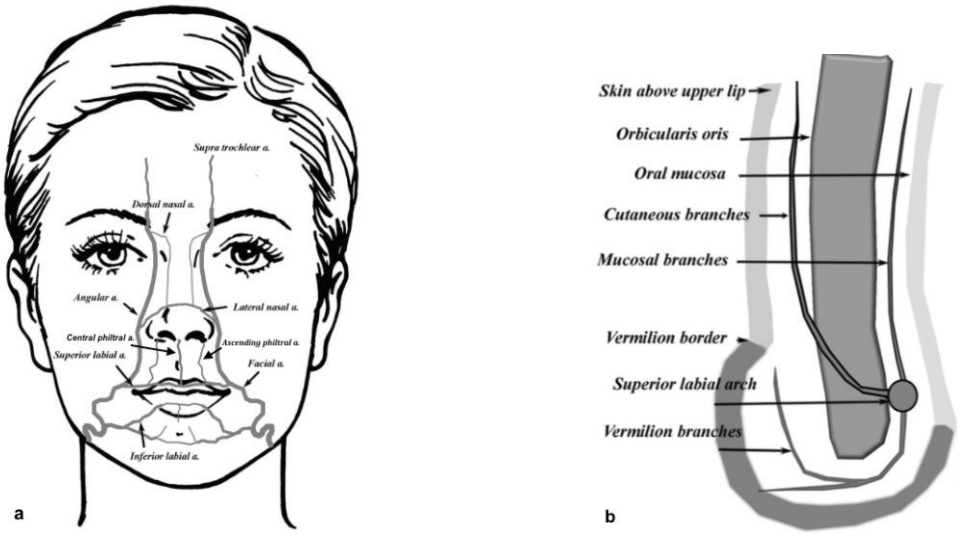


Figure 1.6 Typical anatomy of the labial arteries
Facial arteries (Left), profile view of the upper lip vasculature (right) (Tansatit, T. *et al*, 2014)

Coloured latex dye injected into the cortical arteries of fresh cadavers (aged 62-79) demonstrated that the dye itself does not penetrate into the orbicularis oris muscle, and that it does not progress laterally beyond the philtral columns (Figure 1.6) (Garcia De Mitchell, C.A. *et al*, 2008).

1.2.4 Innervation of the lips

1.2.4.1 Sensory nerve supply

The lips receive their sensory innervation from the trigeminal nerve, which is the fifth cranial nerve. The trigeminal nerve splits into three main branches (Figure 1.7); the ophthalmic (yellow), maxillary (red) and mandibular (blue).

Figure 1.7 Sensory nerve supply to the face
The three main branches of the trigeminal nerve: the ophthalmic (yellow), maxillary (red) and mandibular (blue)

There are a great many nerve endings in the lip, which makes it a highly sensitive area. The superior labial branch of the infraorbital nerve supplies the upper lip, which is a branch of the maxillary nerve. The mental nerve supplies the lower lip, which is a subdivision of the mandibular branch.

1.2.4.2 Motor Nerve Supply

The musculatures of the lips that contribute to facial expression receive innervation from the buccal and marginal mandibular branches of the facial nerve, which is the seventh cranial nerve. In addition, the mandibular branch of the trigeminal nerve has a motor component that supplies the muscles of mastication.

Damage to the trigeminal nerve can affect articulation and prosody (slow rate), whilst damage to the facial nerve can affect articulation (moderate to severe), prosody and facial expressions. In addition, a unilateral incident will cause drooping at the commissure and inability to lift the upper lip during smiling.

1.3 Assessment of the lips

Lip phenotypes can be assessed directly in the clinical setting or by utilising 2D/3D acquisition technique and assessed later. These various methods are summarised (Table 1.2).

1.3.1 Clinical Examination

Traditional anthropometric methods of recording facial variation involve locating anatomical landmarks, and using sliding callipers (or transparent rulers) on the subject's face to measure distances or angles (Farkas, L.G. *et al*, 1992a). As a result, many studies have produced average values of lip parameters, and growth charts (Mehes, K., 1981b, zankl A, E.L., Molinari L, Schinzel A. , 2002. , Zhu, L.Y. *et al*, 2008). Since the advent of high-resolution digital photography and 3D scanning, this is largely performed by landmarking and subsequent computed measurements.

1.3.2 Photography

Photography is the most common clinical method of recording a patient's dental and facial morphology due to the ease of use, relative low cost, and patient safety (Schaaf, H. *et al*, 2009). Photographs are taken routinely for orthodontic patients for the purposes of clinical assessment, treatment planning, and analysing treatment outcomes (Sandler, J. *et al*, 2002). Digital processing has now become the standard format for photographic record keeping in orthodontics (Sandler, P.J. *et al*, 2002). It is important to standardise the image, in terms of head orientation, magnification, lighting and ambient room temperature to provide consistency of image taking between individuals and the same individual over time (Farkas, L.G., 1994).

1.3.3 Lateral cephalogram

Lateral cephalograms of the skull and soft tissues are routinely taken in orthodontics for diagnostic purposes (Broadbent, B.H., 1981). As well as capturing the skeletal structures, it is also possible to view the soft tissue outline, with the addition of an aluminium wedge or barium paste to attenuate the beam. Lateral cephalograms have been used historically to assess growth changes of the lips and surrounding structures (Vig, P.S. *et al*, 1979, Nanda, R.S. *et al*, 1990, Bergman, R.T. *et al*, 2014), and average values of various lip parameters have been derived (Table 1.3).

1.3.4 Cheiloscopy

Cheiloscopy is the study of the patterns of lines and grooves that exist on the vermilion of the lips (Dineshshankar, J. *et al*, 2013). Its main use is in forensic identification, but it has also been described as a method of examining lip variation (Prabhu, R.V. *et al*, 2012). The image is produced by creating a "lip print" of the lips, achieved by painting the lip surface with lipstick and then pressing onto absorbable paper. Lip prints are inexpensive and easy to produce.

Attempts have been made to classify the variation in the appearance of these patterns (Suzuki, K. *et al*, 1970). It has been hypothesised that lip whorls (Table 1.4), may be a sub phenotypic feature in OFC (Neiswanger, K. *et al*, 2009).

1.3.5 Stereo analysis

Stereo analysis is a method of viewing photographs by means of binocular vision, which has the advantage of facilitating depth of perception (Nam, K.W. *et al*, 2012). This occurs due to the phenomenon of parallax, where objects located at different distances from the eye will change their apparent relationship to each other when the eye is moved sideways. Two images of an object are captured using two cameras, placed at some distance apart. The combination of these images produces a 3D representation of the object. The distance of the camera from the object, and the distance between the two cameras affect the depth resolution of the eventual image. However, increasing the separation of the cameras, relative to the distance of the object from the cameras, results in significant distortion, leading to two very different images that cannot be combined succinctly (Halazonetis, D.J., 2007).

1.3.6 Stereophotogrammetry

Stereophotogrammetry is similar to stereo analysis in terms of its arrangement where two cameras are configured as a stereo pair. However, stereophotogrammetry utilises image sequences to capture several images of an object (Heike, C.L. *et al*, 2010). Distances between features can be measured by means of triangulation (Hajeer, M.Y. *et al*, 2004), which involves viewing an object in three-dimensions by means of binocular vision and adding the perception of depth by levelling parallax (Halazonetis, D.J., 2007). The accuracy of measuring facial structures using this method has ranged from 0.5mm – 1.0mm, which is around 1.5% of the total observed variance (Seager, D.C. *et al*, 2009).

1.3.7 Laser scanning

Laser scanning technology utilises a laser beam deflection from a mirror onto the scanning object. The resultant deflection angle is calculated using simple triangulation. As the beam is projected onto the object, it scatters and is captured by a detector. This is then computed and translated into co-ordinates describing different features of the object in the three planes of space (Kau, C.H. *et al*, 2006). The 3D laser scanner measures 20,000 to 120,000 points on an object such as the face to construct a 3D image. It is a non-invasive, non-hazardous technique to both operators and subjects. Although the acquisition time for laser cameras is relatively long (3 seconds), the reliability of this technique is remarkably good over a 3 day period (Kau, C.H. *et al*, 2005b).

Three-dimensional technology has proven successful in a number of recent studies, which evaluated facial changes due to growth (Ferrario, V.F. *et al*, 2000, Kau, C.H. *et al*, 2008). The reliability and accuracy of landmark identification ranges from 0.39 to 1.49 mm with approximately 85% of the landmarks tested accurate to less than 1mm (Toma, A.M. *et al*, 2009). There does not appear to be any significant variation in determining facial landmarks using direct anthropometry, laser scanners and computerised tomography (CT) scanners, however, variation appears to exist between these methods and stereophotogrammetry (Ghoddousi, H. *et al*, 2007, Kook, M.S. *et al*, 2014).

1.3.8 Computerised tomography (CT) and Cone beam computerised tomography (CBCT)

CT is a well-established diagnostic tool, which is used in many areas of medicine. It produces multiple radiographs or slices, which can be reformatted to produce a 3D image. They are generally taken to view internal structures, but can also capture soft tissues and vascular structures. CBCT uses a cone shaped source of ionising radiation, which is directed through the area of interest (Scarfe, W.C. *et al*, 2008). This has the advantage of a shorter capture time and lower radiation dose compared to CT (Kau, C.H. *et al*, 2005a), whilst maintaining good measurement accuracy (Yi, J. *et al*, 2016). Another advantage of CBCT is that the patient is scanned in the upright position, which results in less distortion of the soft tissues in comparison to CT, where the patient is supine. This is particularly useful if the facial soft tissues are reconstructed.

It is not currently feasible to capture the true colour and texture of the skin from a CBCT scan. However, it is possible to manipulate photographic 3D images on to the CBCT scan by image fusion, and several techniques have been described (Khambay, B. *et al*, 2002, Plooij, J.M. *et al*, 2011). As such, CBCT has many useful applications in orthodontics and dentistry (Merrett, S.J. *et al*, 2009, Popat, H. *et al*, 2010).

1.3.9 Magnetic resonance imaging (MRI)

MRI scans use strong magnetic fields and radio waves to produce detailed images of internal structures. Although the scans are not useful for viewing the extra-oral structures off the face, they can be modified to view the intricate underlying facial muscle fibres and their orientations (Olszewski, R. *et al*, 2009, Parker, G.D. *et al*, 2013).

1.3.10 Ultrasound

Ultrasound is a procedure that uses high-frequency sound waves to create an image of internal body structures. As a technology, it is relatively inexpensive, portable and poses no radiation risk.

It is not useful for assessing the extra-oral appearance of the face but there has been some interest in assessing the morphology of the underlying musculature of the lip. It has been hypothesised that defects in the orbicularis oris muscle of the lips, may be a sub-phenotypic feature of OFC, and thus may have a key part to play in the aetiology of OFC (Wijayaweera, C.J. *et al*, 2000, Neiswanger, K. *et al*, 2007).

Table 1.2 Summarising acquisition techniques (Kau, C.H. *et al*, 2010)

Acquisition method	Advantages	Disadvantages
Clinical examination	Inexpensive Non-invasive	Requires all measurements to be taken at once Limited to distances/angles or characteristic features
Photography	Inexpensive Easy to use	Magnification errors Tedious work to map surfaces Pseudo 3D image
Lateral cephalogram radiography	Taken as standard investigation for most orthodontic treatments Reasonable resolution Good correlation to hard tissues	Radiation dose Two-dimensional (2D) image of 3D object Magnification errors
Cheiloscopy	Inexpensive Easy to produce	Limited usage
Stereo analysis	Superior depth perception compared to standard photograph	Three-dimensional (3D) representation from two 2D cameras
Stereophotogrammetry	Multiple motion capture Photorealistic	Low resolution Significant processing capabilities required
Laser scanning	High resolution Quick capture Non-invasive Contour topology and surfaces Medium photorealistic quality	Expensive equipment Technique sensitive
Computerised tomography (CT)	Good measurement accuracy	Expensive equipment High radiation dose Not feasible for multiple exposures Long scan time
Cone beam computerised tomography (CBCT)	Good measurement accuracy Quick capture Less expensive Reduced radiation dose	Not feasible for multiple exposures Requires radiological reporting
Magnetic resonance imaging (MRI)	Non-invasive	Internal structures only Long capture time
Ultrasound	Non-invasive Inexpensive Portable	Internal structures only

1.4 Methods of phenotyping

There are essentially three approaches to phenotyping the lips. Firstly, anthropometric (measuring distances between anatomical landmarks), secondly morphometric (incorporating shape, size and orientation) and finally a full description and characterisation of lip phenotypes (Figure 1.8).

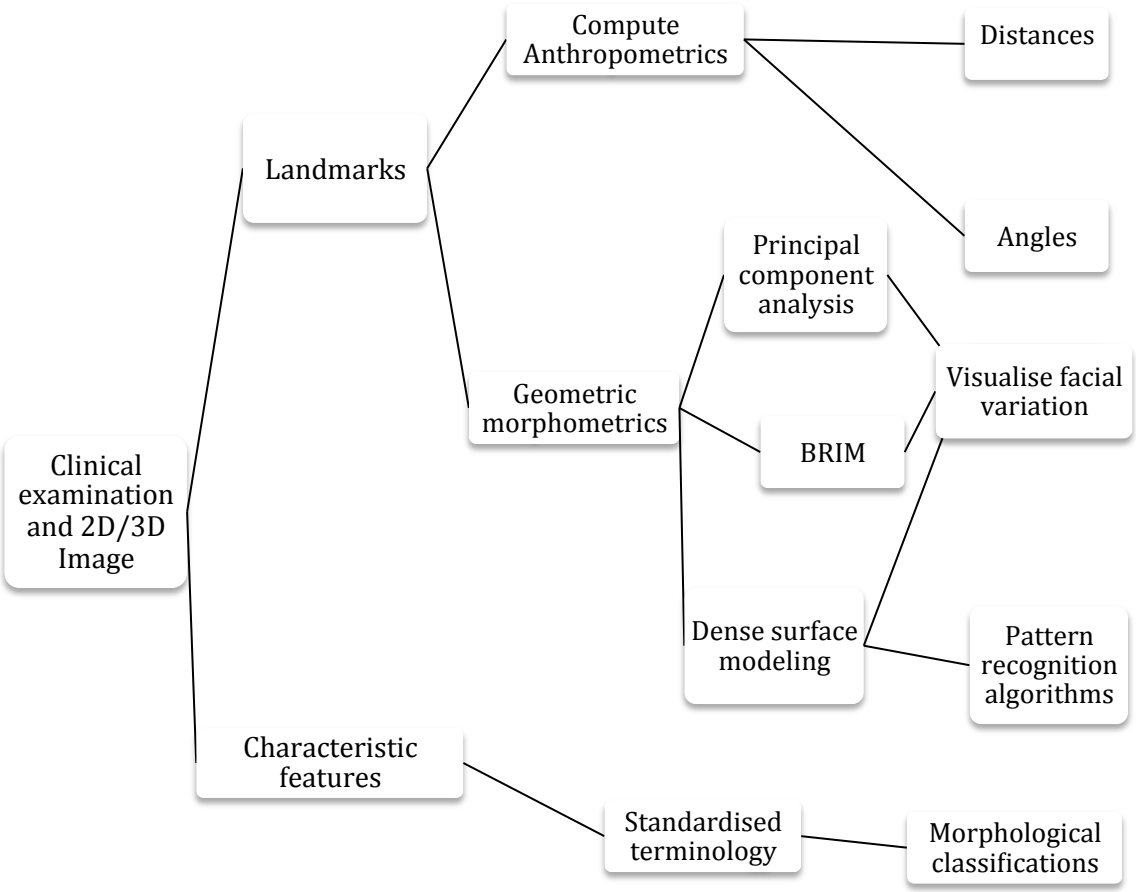


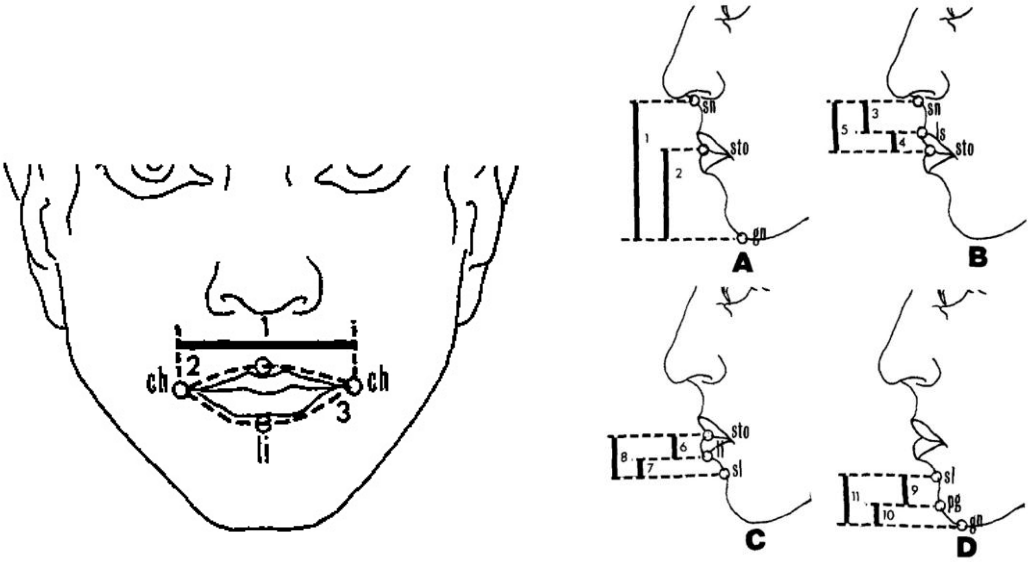
Figure 1.8 Methods of quantifying and describing lip phenotypes

1.4.1 Anthropometrics

Anthropology is the study of the measurement of clinical features, using the various landmarks. Measurements should be performed with the patient relaxed and in a neutral head position, as facial expression can affect the ability to accurately locate landmarks.

1.4.1.1 Measurements of the lips

Measurements are made based on the distance between landmarks (Figure 1.9 and 1.10). Using this methodology, average values can be derived from clinical examination, lateral cephalograms and 3D images (Table 1.3).



Landmarks		Measurement
Vertical		
1	sn-gn	Height of the lower face
2	sto-gn	Height of the lower third of the face
3	sn-ls	Height of the cutaneous lip (philtral length)
4	ls-sto	Height of the upper vermilion
5	sn - sto	Height of the upper lip
6	sto-li	Height of the lower vermilion
7	li-sl	Height of the cutaneous lower lip
8	sto-sl	Height of the lower lip
9	sl-pg	Height of the upper chin
10	pg-gn	Height of the lower chin
11	sl-gn	Height of the entire chin
Horizontal		
12	chL-chR	Length of the labial fissure (mouth width)
13	ch-ls-ch	Upper vermilion arc
14	ch-li-ch	Lower vermilion arc

Figure 1.9 Anthropometric measurements of the lip region (Farkas, L.G. *et al*, 1984)

The average fullness of the lips can be recorded using several different methods; a clinical measurement from the frontal view (ls-sto) measures the height of the vermilion, measurements taken from lateral cephalogram measures the thickness from the internal surface to the exterior surface. It is also possible to measure the volume of the lips using 3D techniques. As a result of this, it can be difficult to draw comparisons from different studies, due to the varying methods.

1.4.2 Morphometrics

Morphometrics is the quantitative summary of size, shape and orientation differences among organisms or their components (Bookstein, F.L., 2001). Geometric morphometrics is a means of quantitative analysis, via a method of recording co-ordinates rather than linear variables (Bookstein, F.L., 1997, Ivan Perez, S. *et al*, 2006). Co-ordinates can be located via specific landmarks or multiple intermediate landmarks. Shape variation is then visually analysed (Klingenberg, C., 2013).

1.4.2.1 Principal component analysis (PCA)

PCA is a tool for explanatory data analysis; it is a statistical method of simplifying a set of landmarks or observations into a reduced set of related variables, to explain shape variation. Using 21 landmarks of the face (Farkas, L.G., 1994), analyses have been performed in order to visualise and explain facial variation within the population (Figure 1.10). Fourteen PC's were produced, which explained 82% of the total variance. The first four relate to 51.2% of the variance.

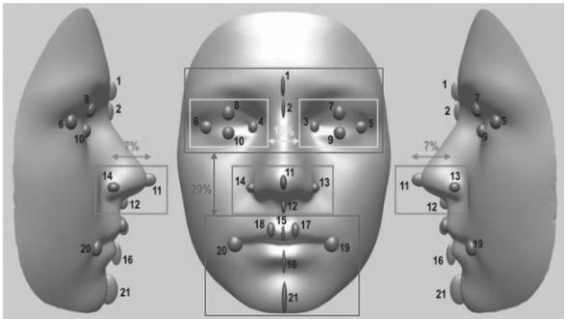


Figure 1.10 Standard deviation envelopes highlighting facial variation (Toma, A.M. *et al*, 2012)
PC1 – (Red) Variation face height, PC2 – (Yellow) Variation inter-eye width, PC3 – (Green) Prominence of the nose, PC4 – Protrusion of the upper lip relative to the chin (skeletal pattern)

This method has been used extensively in the literature to attempt to describe facial variation, and has been successful in phenotype/genotype association (Paternoster, L. *et al*, 2012)

Table 1.3 Average measurements of lip phenotypes

Age	Sample	Population	Lengths (mm)		Method	Reference
			Male	Female		
Height of the lower face (sn-gn)						
18-25	90 (50m, 40f)	North America	70.4+/-6.6	63.0 +/- 4.3	C	(Farkas, L.G. *et al*, 1984)
Height of the lower third of the face (sto-gn)						
18-25	100 (50m, 50f)	North America	48.8+/- 4	44.8 +/-3.4	C	(Farkas, L.G. *et al*, 1984)
Medial height of the cutaneous upper lip (philtral length) (sn-ls)						
15	2,500	Central Europe	17.5	16.5	C	(Zankl, A. *et al*, 2002)
15	32 (23m, 9f)	North America	15.1 +/- 2.2	13.3 +/- 1		(Farkas, L.G. *et al*, 1992b)
18-25	89 (50m, 39f)		16.7+/- 2.2	13.3 +/- 2.1		(Farkas, L.G. *et al*, 1992b)
Medial vermilion height of the upper lip						
15	101 (50m, 51f)	North America	9.3+/-1.5	8.8+/-1.3	C (ls-sto)	(Farkas, L.G. *et al*, 1992b)
18-25	89 (50m, 39f)		7.4 +/- 1.7	7.7 +/- 1.1		(Farkas, L.G. *et al*, 1984)
16	32 (16m, 16f)		15.97 (1.72)	12.96 (2.1)	LC (a-p thickness)	(Mamandras, A.H., 1988)
Adult	46 (20m, 26f)		14.8 +/-1.4	12.6 +/- 2		(Arnett, G.W. *et al*, 1999)
16	32 (16m, 16f)		4.0+/- 0.5	3.3 +/- 0.6	LC (cm2)	(Mamandras, A.H., 1988)
21-49	70 (38m, 32f)	England, UK	2.8 (0.8)	2.3 (0.8)	3DS (cm2)	(Sawyer, A.R. *et al*, 2009)
Philtral width (cph-cph)						
Infants	40	North America	7.1 +/- 1.1		C	(Franz, M.L. *et al*, 1972)
5-6	109 (51m, 58f)	Japan	8.7+/-1.4	8.6+/-1.3		(Mori, A. *et al*, 2005)
Philtral depth (cph-cph → deepest point at midline)						
24-39	55 (25m, 30f)	Japan	1.23+/-0.58	0.88+/-0.45	3DL	(Kishi, N. *et al*, 2012)
Nasolabial angle (pr-sn-ls)						
18-25	89 (50m, 39f)	North America	99+/-8.0	99+/-8.7	C	(Farkas, L.G. *et al*, 1992b)
5-6	109 (51m, 58f)	Japan	95.2°	96.8°	C	(Mori, A. *et al*, 2005)
Medial vertical upper lip length (sn-sto)						
13-15	32	North America	23.8 +/- 1.5	20.1 +/- 2	LC	(Burstone, C.J., 1967)
15	88 (42m, 46f)		23 +/- 2.5	21 +/- 2.4	C	(Peck, S. *et al*, 1992)
15	101 (50m, 51f)		22.3 +/- 2.1	20.1 +/- 2	C	(Farkas, L.G. *et al*, 1992b)
15	50 subjects	England, UK	25.93 +/- 3.98		LC(MxP- sto)	(Vig, P.S. *et al*, 1979)
16	32 (16m, 16f)	North America	21.16 (1.75)	18.92 (2.5)	LC(Pal– sto)	(Mamandras, A.H., 1988)
18-25	90 (50m, 40f)		22.7 +/-2.3	19.6 +/- 2.1	C	(Farkas, L.G. *et al*, 1984)
21-49	70 (38m, 32f)	England, UK	21.3	19.2	3DS	(Sawyer, A.R. *et al*, 2009)
Adult	46 (20m, 26f)	North America	24.5 +/- 2.5	21.0 +/- 2	LC	(Arnett, G.W. *et al*, 1999)
Medial vertical lower lip length (sto-gn)						
13-15	32	North America	49.9 +/-4.5	46.4 +/- 3	LC	(Burstone, C.J., 1967)
18-25	89 (50m, 39f)	North America	48.8 +/- 4	44.8 +/- 3	C	(Farkas, L.G. *et al*, 1984)
15	50	England, UK	44.07 +/- 4.23		LC (MxP – sto)	(Vig, P.S. *et al*, 1979)
16	32 (16m, 16f)	North America	44.07 (3.2)	39.81 (3.6)		(Mamandras, A.H., 1988)
Adult	46 (20m, 26f)	North America	54.3 +/- 2.4	46.9 +/- 2	LC (LLS –Me)	(Arnett, G.W. *et al*, 1999)
Medial vermilion height of the lower lip						
18-25	89 (50m, 39f)	North American	8.8 +/- 2.0	9.0 +/- 1.5	C (sto-li)	(Farkas, L.G. *et al*, 1984)
16	32 (16m, 16f)	North America	12 (1.64)	10.9 (1.91)	LC (a-p thickness)	(Mamandras, A.H., 1988)
Adult	46 (20m, 26f)	North America	15.1+/-1.2	13.6 +/- 1		(Arnett, G.W. *et al*, 1999)
16	32 (16m, 16f)	North America	5.6 +/- 0.5	5.3 +/- 1.0	LC (cm2)	(Mamandras, A.H., 1988)
21-49	70 (38m, 32f)	England, UK	2.9 (0.8)	2.3 (0.8)	3DS (cm2)	(Sawyer, A.R. *et al*, 2009)
Mouth width (ch-ch)						
5-6	109 (51m, 58f)	Japan	37.6+/-2.9	35.7+/-2.3	C	(Mori, A. *et al*, 2005)
18-25	100 (50m, 50f)	North America	54.1 +/- 3.8	50.6 +/- 3.1	C	(Farkas, L.G. *et al*, 1984)

C = clinical measurements, LC = Lateral cephalogram 3DS = 3D stereophotogrammetry

1.4.2.2 Bootstrapped response-based imputation modelling (BRIM)

This method firstly involves locating five positioning landmarks on the face to establish a rough orientation (Figure 1.11a). A symmetrical mirror image of the face is then produced (Figure 1.11b), under the assumption that asymmetry is a by-product of environmental disturbances rather than genetic (Windhager, S. *et al*, 2014).

a) Initial **b) Reflected** **c) Following BRIM**

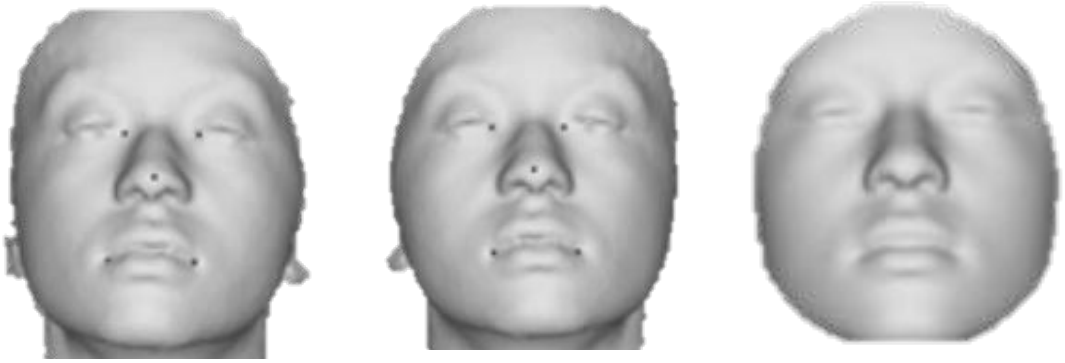


Figure 1.11 Method of BRIM (Claes, P. *et al*, 2014)

Next, a mesh containing 7,150 quasi landmarks is automatically mapped to the subject's scanned 3D image. The image is processed further to produce a symmetrised reconstructed image (Figure 1.11c). PCA was then performed which produced 44 principal components, which summarised 98% of the total variation. Colour maps were then used to visualise facial variation (Claes, P. *et al*, 2014)

1.4.3 Classification by characteristic shape

Certain features cannot be quantified by measurements alone, as some deviations arise as variations in shape or texture, rather than lengths or angles. These variations are described by classification of characteristic shapes (Table 1.4).

It is also possible to classify the depth of the philtrum using the Likert scale for lips (Figure 1.12), which was originally developed as a diagnostic aid for detecting foetal alcohol syndrome (FAS). The scale varies from a deep philtrum shape, with a thick upper lip and exaggerated Cupid's bow shape (1) to a progressively shallower (2-4) to a smooth philtrum, thin upper lip and flat Cupid's bow shape (Astley, S.J. *et al*, 1999). In a study of 84 subjects without FAS, only 4 individuals had a smooth philtrum and only 5 had a very thin upper lip; whilst 41 individuals with FAS had a smooth philtrum and 25 had very thin lip (Astley, S.J. *et al*, 1996). It is suggestive that these features occur commonly in individuals with FAS, and forms part of the diagnostic procedure.

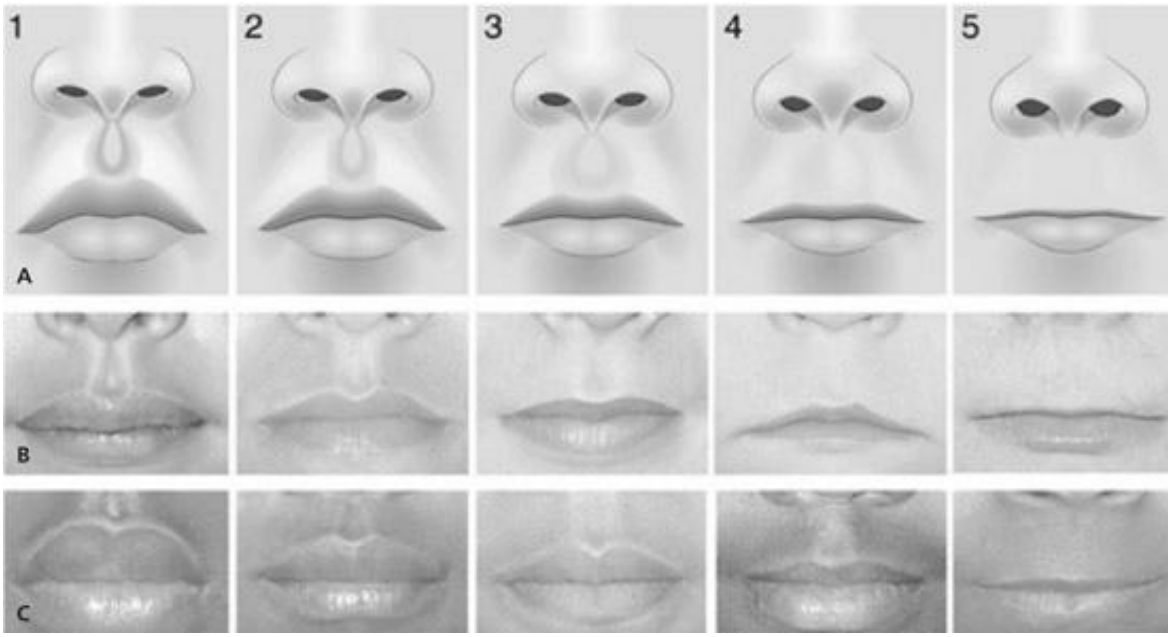
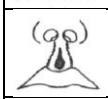
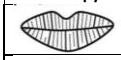
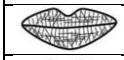


Figure 1.12 Likert scale of upper lip fullness and philtral depth
Diagrammatic representation (top row), Caucasian scale (middle row), Afro-Caribbean scale (bottom row) (Astley, S.J. *et al*, 1999)

The height of the vermilion of the lower lip varies among ethnic groups, and the vermilion should be compared to a population of same ethnic background (Astley, S.J. *et al*, 1995).

Table 1.4 Characteristic features of the lip region

Feature	Definition	Sample & Population	Prevalence	Reference
Philtrum length				
Short	Apparently decreased distance			(Hennekam, R.C. *et al*, 2009)
Long	Apparently increased distance			
Philtrum width				
Narrow	Apparently decreased distance between philtral ridges			(Franz, M.L. *et al*, 1972, Hennekam, R.C. *et al*, 2009)
Wide	Apparently increased distance			
Philtrum depth				
	Smooth: Flat skin surface, the philtral columns have almost no prominence	84 controls (birth-27yrs) North America	4.8%	(Astley, S.J. *et al*, 1996)
		109 (51m, 58f) 5-6yrs, Japan	13% M=F	(Mori, A. *et al*, 2005)
	Parallel: The philtral columns originate from the nostril sills, and exhibit an almost parallel shape		42% (F>M)	
	Triangular / downward divergent: Prominence of a triangular philtrum with the apex to the columella		33% (F>M)	
	Convex - The upper cross of the philtral columns is located in the middle of the upper lip. No philtrum dimple in the upper half of the upper lip		12% (M>F)	
Cupid's bow				
Absent	Lack of paramedian peaks and median notch of the upper lip vermilion			(Carey, J.C. *et al*, 2009)
Exaggerated	More pronounced paramedian peaks and median notch of the Cupid's bow			
Upper lip				
Thin	Apparently reduced height of the vermilion of the upper lip in the frontal view	84 controls (birth-27yrs) North America	Likert 5:6% Likert 4:41%	(Astley, S.J. *et al*, 1996, Carey, J.C. *et al*, 2009)
Thick	Apparently increased height of the vermilion of the upper lip on the frontal view		Likert 1:13% Likert 2:16%	
Everted	Inner aspect of the upper lip vermilion (oral mucosa) visible in a frontal view			(Carey, J.C. *et al*, 2009)
Tented	Triangular appearance of the oral aperture with the apex in the midpoint of the upper vermilion and the lower vermilion forming the base			
Cheiloscopy				
	Straight Vertical - Clear-cut vertical grooves	60 (30m, 30f) 20-30yrs India	(M)6.6% (F)43.3%	(Nagalaxmi, V. *et al*, 2014)
	Branching - branching Y-shaped pattern		(M)8.3%% (F)28.3%,	
	Intersecting grooves		(M)46.6% (F)11.6%	
	Diamond & reticular		(M)26.6% (F)3.3 %	
	Lower lip whorls	North America & Hungary 142 (48m, 86f)	2.3%	(Neiswanger, K. *et al*, 2009)
Commissures				
Upturned	Oral commissures positioned superior to the midline labial fissure			(Carey, J.C. *et al*, 2009)
Downturned	Oral commissures positioned inferior to the midline labial fissure			
Lip-Chin shape				
	Flattened	89 (50m, 39f) 18-25 years North America	0	(Farkas, L.G. *et al*, 1984)
	Shallow and curved		15/50 (m) 13/39 (f)	
	Deep and curved		45% (F>M) 28% (M) 67% (F)	
	Deep and indented		36% (M>F) 42% (M) 28% (F)	
Chin dimple				
No dimple		Germany	82%	(Ritz-Timme, S. *et al*, 2011)
		Italy	80%	
		Lithuania	71%	
Dimple		Germany	18%	
		Italy	20%	
		Lithuania	29%	
Skeletal pattern				
Skeletal I	Balanced facial profile	1000 European	40%	(Foster, T.D. *et al*, 1974)
Skeletal II	Relative mandibular retrognathia		56%	
Skeletal III	Relative mandibular prognathia		5%	
		Chinese	12%	(Lew, K.K. *et al*, 1993)

1.4.3.1 Reported associated features

Some features are reportedly associated with each other (Table 1.5, represented as grey squares); however, this is based on expert opinion rather than epidemiological studies (Hennekam, R.C. *et al*, 2009, Carey, J.C. *et al*, 2009).

Table 1.5 Associated lip phenotypes

		Philtrum				Cupid's bow		Upper lip
		Smooth	Deep	Wide	Long	Absent	Exaggerated	Thin
Philtrum	Smooth							
	Deep							
	Wide							
Cupid's bow	Absent							
	Exagger-ated							
Upper lip	Thin							

1.5 Summary

The current methods of phenotyping lip phenotypes have their limitations. Phenotyping according to landmarking anatomical locations and measuring distances is restricted to the number of landmarks available. In addition, some soft tissue points are less reliable than others, and can be difficult to attain, which could potentially lead to misleading results (Mehes, K., 1981a, Hennekam, R.C. *et al*, 2009). Also measurements of small distances (<10cm) and difficult to define landmarks, such as philtrum width; have been shown to be inherently unreliable (Ward, R.E. *et al*, 1991). Locating landmarks on 3D images is advantageous, as it has been shown to be an accurate and reproducible (Toma, A.M. *et al*, 2009) method. However, by limiting the data to principal components, it tends to oversimplify shape variation that may not reflect biological craniofacial development. Geometric morphometrics attempts to overcome this shortfall by adding semi-landmarks between two distant landmarks, however, this method still fails to record other features that have been shown to exist. Characterising features on the other hand gives us more information about the textural and shape variations that occur as well as features that are either present or not; however, this technique is currently limited in the scientific literature to only a few features.

Chapter 2: Lip development

2.1 Introduction

This chapter explores existing theories of normal and abnormal lip development including genetic and environmental mediators.

2.2 Normal lip development

The existing understanding of the mechanism of lip development is largely based upon studies of histological sections taken at various stages of embryological development. At the molecular level, animal models have identified specific roles for several major signalling pathways important in co-ordinating normal lip morphogenesis. In addition, several genetic studies have identified many genes involved in CLP pathogenesis.

2.2.1 Mechanism of lip development

Histological studies have involved the assessment of humans, mice, chicks or cynomolgus monkeys foetuses (Trasler, D., 1968, Hinrichsen, K., 1985, Senders Cw, P.E., Hendrickx Ag, Cukierski Ma, 2003). Whilst obvious ethical issues exist for the assessment of human foetuses, there are limitations to assessing the development of the lip region in other animals, which have differing morphology to humans.

Development of the human face begins around the fourth week of gestation (O'rahilly, R., 1972), with upper lip development commencing at day twenty-four (Jiang R, B.J.O., Lidral A.C., 2006). At this point, migrating neural crest cells combine with mesodermal cells, to create the facial primordia.

At stage 12 (26 days), the primitive mouth (stomodeum) is bound by five prominences (Figure 2.1); the frontonasal prominence, two maxillary prominences and two mandibular prominences. The paired mandibular processes fuse at the midline.

During stages 13-14 (4-5weeks) nasal pits form, as the frontonasal process gives rise to the medial and lateral nasal processes. There is subsequent rapid mesenchymal growth at the maxillary and medial nasal processes, which pushes the nasal pits medially.

During stages 15-16 (sixth week), the nasal prominences merge. Fusion between the lateral and medial nasal processes starts posterior to the nasal pits and then proceeds

in an anterior direction (Trasler, D., 1968, Hinrichsen, K., 1985) This region subsequently fuses to the maxillary prominences, which form the lateral parts of the upper lip (Sun D, B.S., Hay Ed. , 2000, Cox, T., 2004).

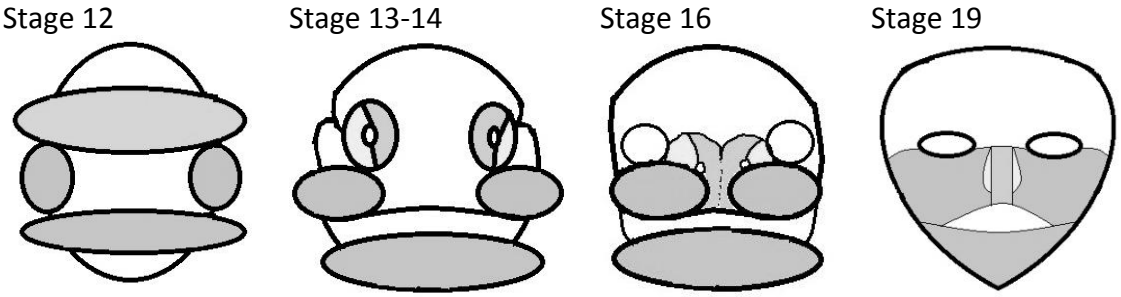


Figure 2.1 Stages of human upper lip development, adapted from (Dixon, M.J. *et al*, 2011)

Frontonasal prominence (green), medial nasal process (blue), lateral nasal process (yellow), maxillary process (purple), mandibular process (pink)

2.2.2 Molecular pathways involved in lip development

A complex network of transcription factors and signalling molecules tightly regulate craniofacial development (Jiang R, B.J.O., Lidral A.C., 2006), including neural crest formation, migration, patterning, proliferation, and apoptosis (Senders Cw, P.E., Hendrickx Ag, Cukierski Ma, 2003).

Many signals and genes have been shown to play an important role in facial morphogenesis by controlling the development of facial prominences and the skeletal structure of the face (Table 2.1). Components of several signalling pathways are expressed during craniofacial development and gene knockout studies in mice have confirmed the involvement of these pathways in upper lip morphogenesis (Hu D, H.J., 1999, Ashique, A.M. *et al*, 2002, Liu, W. *et al*, 2005, Jiang R, B.J.O., Lidral A.C., 2006).

2.2.2.1 The *Bmp* pathway

Bone morphogenetic proteins (*Bmp*) are a group of signalling molecules secreted by the transforming growth factor beta (*Tgfβ*) superfamily. Many animal studies have demonstrated that they play an important role in regulating a diverse number of

developmental processes and are function in the evolution of facial shape and size (Wan, M. *et al*, 2005).

In the developing chick facial primordial, *Bmp4* is highly expressed in the distal epithelia of the medial and lateral nasal, maxillary and mandibular processes (Wan, M. *et al*, 2005). It has also been demonstrated that ectopic application of either *Bmp2* or *Bmp4* proteins induces overgrowth and produces changes to the patterning of the facial primordia (Barlow, A.J. *et al*, 1997). Whilst, inhibiting *Bmp* signaling by application of *Noggin* (a specific *Bmp* antagonist) causes reduced mesenchymal proliferation and outgrowth (Ashique, A.M. *et al*, 2002). This effect has also been replicated in fish and birds (Wan, M. *et al*, 2005).

Msx1 and Msx2 are downstream transcription factors of the Bmp pathway (Barlow, A.J. *et al*, 1997), and both are likely play critical roles in facial mesenchymal proliferation. In mouse embryos, *Bmp4* is expressed in the stomodeum prior to and during lip fusion (Gong, S.G. *et al*, 2003) whereas *Msx1* and *Msx2* are expressed in the adjacent facial mesenchyme. Alterations in the function or missense mutations of the *MSX1* gene have been associated with CL/P and tooth agenesis in humans (Jezewski, P.A. *et al*, 2003, Suzuki, Y. *et al*, 2004). However, in mouse models, those deficient in *Msx1* exhibit CP, but not CLP (Satokata, I. *et al*, 1994), whilst mice lacking in both *Msx1* and *Msx2* exhibit bilateral CLP (Jiang R, B.J.O., Lidral A.C., 2006). In knockout mouse models, *Msx1−/−* mutant mice have shortened maxilla and mandibles as well as defects in palatal mesenchyme proliferation (Satokata, I. *et al*, 1994, Zhang, Z. *et al*, 2002). These studies indicate that *Bmp4* and *Msx1/Msx2* function together in a common molecular pathway, which is essential for normal facial growth and upper lip morphogenesis (Jiang R, B.J.O., Lidral A.C., 2006).

Transforming growth factors (*TGF*) are associated with extra-cellular matrix defects (Tgfα) and differentiation defects (*Tgfβ*). Their locus has been associated with NSCL/P in some human populations (Schutte, B.C. *et al*, 1999), and mice studies suggest *Tgfβ* has an important role during fusion of the secondary palate (Cobourne, M.T., 2004)

2.2.2.2 The *Fgf* pathway

Fibroblast growth factors (*Fgf*) and their cell surface receptors (*Fgfr*) make up a large and complex family of signaling molecules that play important roles in a variety of processes of embryogenesis and tissue homeostasis (Johnston, M.C. *et al*, 1995,

Bachler, M. *et al*, 2001, Stanier, P. *et al*, 2012), as well as an essential role in midfacial growth and upper lip development (Jiang R, B.J.O., Lidral A.C., 2006).

Fgf8 is expressed in the frontonasal, maxillary and mandibular epithelia during active facial primordial outgrowth (Bachler, M. *et al*, 2001) and has the capability to maintain mesenchymal gene expression, regulating facial primordial outgrowth (Neubuser, A. *et al*, 1997). In knockout mice studies, inactivation of *Fgf8* in the mandibular epithelium demonstrated that it is required for mandibular mesenchymal survival and patterning (Trumpp, A. *et al*, 1999), whereas in the forebrain and facial ectoderm, inactivation led to severe facial defects, including midfacial cleft (Firnberg, N. *et al*, 2002).

The fibroblast growth factor receptors *Fgfr1* and *Fgfr2* also play essential roles in the signaling of neural crest migration, survival, proliferation, and patterning of both the facial epithelia and mesenchyme (Trokovic, N. *et al*, 2003). Nonsense mutations and deletions in the *FGFR1* gene causes Kallmann syndrome, an autosomal dominant disorder characterised by infertility and anosomia, in which 5% of individuals also have CLP (Dode, C. *et al*, 2003).

2.2.2.3 The *Shh* pathway

The sonic hedgehog (*Shh*) pathway plays an important role in many mechanisms, including morphogenetic patterning activity, including left-right axis establishment, dorsoventral patterning of the neural tube and craniofacial development (Ingham, P.W. *et al*, 2001, Lettice, L.A. *et al*, 2003, Kurosaka, H., 2015). It also plays an aetiological role in many human diseases, particularly holoprosencephaly, cancer and CLP (Roessler, E. *et al*, 2003, Kurosaka, H. *et al*, 2014, Kurosaka, H., 2015). The Shh pathway also activates *Ptch1* and members of the *Gli* family of transcription factors through a complex of indicators (Ingham, P.W. *et al*, 2001, Jiang R, B.J.O., Lidral A.C., 2006). Mutations in *PTCH1* causes bilateral CLP (Jeong, J. *et al*, 2004, Kurosaka, H. *et al*, 2014), and is associated with Gorlin-Goltz syndrome (Larsen, A.K. *et al*, 2014).

Shh is expressed in the ectoderm of the facial primordial and its signaling is important for mesenchymal proliferation and survival initially and later craniofacial growth (Jeong, J. *et al*, 2004), mutations or inhibitions in *Shh* cause severe cranial deficiencies (Hu D, H.J., 1999). In animal studies, inhibition in the cranial mesenchyme caused neural crest mesenchymal cell death (Ahlgren, S.C. *et al*, 1999), and specific inactivation of *Smo* further confirms that *Shh* signaling is required for both survival and proliferation of the facial mesenchyme (Jeong, J. *et al*, 2004). Inhibition of *Shh* in chick

frontonasal processes caused inhibited facial outgrowth and CL, whilst ectopic application of caused mediolateral expansion of that tissue (Hu D, H.J., 1999).

2.2.2.4 The RA pathway

Retinoid acids (RA) regulate many events in patterning during development, including cell proliferation, differentiation and apoptosis (Gudas, L.J. *et al*, 2011). Studies have shown that RA play important roles during palate development, and in addition, excess RA causes CP in foetuses of both rodents and humans (Okano, J. *et al*, 2014). Mice harboring mutations in both the retinoic acid receptor genes *RARα* and *RARβ* display a severe median cleft and defects in other neural crest-derived structures (Johnston, M.C. *et al*, 1995).

2.2.2.5 The *Wnt* pathway

The *Wnt* glycoproteins regulate cell proliferation, cell fate determination, differentiation and cell survival. Mice studies have identified roles for *Wnt3* and *Wnt9b* during mouse embryogenesis; both are expressed in the ectoderm of the developing facial primordia (Lan, Y. *et al*, 2006), and canonical *Wnt* signaling is specifically activated in the epithelia and underlying mesenchyme in the medial nasal, lateral nasal, and maxillary processes prior to fusion (Merrill, B.J. *et al*, 2004).

The canonical *Wnt* pathway signals through β-catenin, a dual functional protein involved in cell adhesion and signaling. As β-catenin enters the nucleus, it activates the *Tcf/Lef* family of transcription factors and regulates transcription of downstream genes (Zhu, X.J. *et al*, 2016). The pathway also has an important role in regulating *Shh* signaling during cranial nerve development (Kurosaka, H. *et al*, 2015), and overlaps with *Bmp4* during facial development, regulating midfacial morphogenesis (Gong, S.G. et al, 2003, Liu, W. et al, 2005).

Mouse studies have demonstrated associations of a nonsense mutation in the *WNT3* gene with tetra-amelia, a rare recessive genetic disorder characterised by complete absence of all four limbs and CLP (Niemann, S., 1993, Juriloff, D.M. *et al*, 2001). A targeted mutation in the *Wnt9b* gene also caused severe kidney developmental defects and an incomplete penetrance of CLP (Carroll, T.J. *et al*, 2005).

Inactivation of *Lrp6*, a co-receptor of the *Wnt/β-catenin* signaling pathway, leads to CLP in mice (Song, L. *et al*, 2009).

2.2.2.6 The Egf pathway

Epidermal growth factor (*EGF*) stimulates cell growth, proliferation and differentiation by binding to *EGFR*. *EGFR* signalling is necessary for normal craniofacial development and is mediated in part by its downstream targets, the MMPs. Knockout mice lacking in *Egfr* exhibited a low penetrance of CLP (Miettinen, P.J. et al, 1999), whereas the *TGFα* locus has been associated with NSCL/P in some human populations (Cobourne, M.T., 2004).

2.2.2.7 The Pdgf pathway

Platelet derived growth factor (PDGF). Autonomous to the neural crest, because conditional disruption of *Pdgf* in neural crest cells results in a similar facial cleft. Mice carrying a null mutation in *Pdgfr* and mice homozygous for mutations in both the *Pdgfa* and *Pdgfc* genes have a median cleft (Ding, H. et al, 2004).

2.2.2.8 Other genes and pathways

There are many other genes that have been implicated in upper lip development, including several which have been associated with SCL/P (Table 2.2). Many of these, including *PVRL1, P63, IRF6*, and *CDH1* are predominantly expressed in epithelial tissues, indicating that proper epithelial differentiation, organisation, or patterning play important roles in lip development (Jiang R, B.J.O., Lidral A.C., 2006).

Table 2.1 Signalling pathways thought to have an important role during lip development

Pathways	Main role in lip development		Function	Genes	Animal Study findings
Bone morphogenetic proteins (*BMP*)	Initiation and growth of the facial prominences	Promotes facial primordial outgrowth	Regulate diverse developmental processes, including cell proliferation, differentiation, apoptosis, and tissue morphogenesis	*Bmp2* *Bmp4*	Induces growth and controls the patterning of the chick facial primordia (Barlow, A.J. *et al*, 1997)
					Inhibition of Bmp (by application of *Noggin*, a specific Bmp antagonist), in the chick facial primordia caused reduced mesenchymal proliferation and outgrowth (Ashique, A.M. *et al*, 2002)
			Regulates facial primordial outgrowth Studies indicate that *Fgf* signalling plays essential roles in midfacial growth and upper lip development		Possible downstream activators of *msx1* and *msx2* (Barlow, A.J. *et al*, 1997)
			Overlapping expression of homeobox genes		Role in mandibular development (Liu, W. *et al*, 2005)
					Role in CL (elevated apoptosis, delayed/defective lip fusion) (Liu, W. *et al*, 2005)
Fibroblast growth factors (*FGF*) and their cell surface receptors (*FGFR*)		Tissue homeostasis – regulating primordial outgrowth	Studies indicate that *Fgf* signalling plays essential roles in midfacial growth and upper lip development (Jiang R, B.J.O., Lidral A.C., 2006)	22 *Fgf* genes	
				Fgf8	Expressed in the frontonasal and mandibular epithelia during active facial primordial outgrowth (Bachler, M. *et al*, 2001). May stimulate mesenchymal proliferation – regulating facial primordial outgrowth
				FGFR1 & FGFR2	Associated with craniosynostosis syndromes (Hollway, G.E. *et al*, 1997) where CP in a broad phenotype (Stanier, P. *et al*, 2012)
Sonic hedgehog family (*SHH*)		Proliferation defects: Ectodermal signal that regulates outgrowth and fusion	Mediates ectodermal functions. Left–right axis establishment, dorsoventral patterning of the neural tube, endoderm development, limb and craniofacial development, brain and pituitary development, holoprosencephaly (Kurosaka, H., 2015)		Mice mutations had hypoplastic nasal process outgrowth, epithelial seam persistence and CL. (Kurosaka, H. *et al*, 2014) Enhanced SHH restricts WNT pathway (Kurosaka, H. *et al*, 2014)
Retinoic acid pathways		Regulation of development, differentiation and apoptosis		*RARα* *RARb*	Mice harbouring mutations display a severe median cleft and defects in other neural crest-derived structures (Johnston, M.C. *et al*, 1995)
Canonical Wnt pathway	Fusion	Regulate diverse developmental processes, including cell proliferation, cell fate determination and differentiation, and cell survival	A dual functional protein involved in cell adhesion and signalling Directly regulates facial mesenchymal growth and lip fusion	*ß-catenin*	Cell adhesion and signalling
					In mice, WNT inhibition affects *IRF6* signalling, resulting in increased proliferation and decreased apoptosis (Kurosaka, H. *et al*, 2014)
Epidermal growth factor (Egf)		Role in growth, proliferation and differentiation of numerous cell types		*Egf*	Mice lacking *Egfr* exhibit a low penetrance of CL/P and residual epithelium in the midline (Miettinen, P.J. *et al*, 1999)
Transforming growth factor (*TGF*)		Extra-cellular matrix defects (Tgfα) and Differentiation defects (*Tgfß*)	Locus has been associated with NSCL/P in some human populations (Schutte, B.C. *et al*, 1999)	*Tgfα*	
				Tgfß	Mice studies suggest important role during fusion of the secondary palate (Cobourne, M.T., 2004)
Platelet derived growth factor (PDGF)			Autonomous to the neural crest, because conditional disruption of *Pdgf* in neural-crest cells results in a similar facial cleft	*Pdgf*	Mutations in *Pdgfr* result in a median cleft in mice (Ding, H. *et al*, 2004)

2.2.3 Fusion of the prominences

Initial merging of the prominences is thought to occur as a result of epithelial bridging via desmosomes (Hinrichsen, K., 1985, Sun D, B.S., Hay Ed. , 2000). This involves a series of cellular transformations, whereby cells undergo programmed cell death in order to promote epithelial adherence by exposing the basal layers and thus forming adherence to the underlying layers (Jiang R, B.J.O., Lidral A.C., 2006). Epithelial seams appear to subsequently disintegrate, and the merged processes transform into a continuous mesenchymal structure (Sun D, B.S., Hay Ed. , 2000).

Following fusion, the maxillary processes continue to grow rapidly and push the nasal pits and medial nasal processes medio-frontally (Hinrichsen, K., 1985). The groove between the medial nasal prominences eventually becomes smooth due to continued growth and confluence of the mesenchyme.

Lip development is complete by stage 19 (48 days). It is accepted that during embryological development, the frontonasal process forms the intermaxillary segment resulting in both the philtrum and primary palate and the maxillary process fuses with the medial and lateral nasal processes to form the lateral part of the upper lip (Figure 2.1, Stage 19).

2.2.4 Summary

Upper lip development encompasses a sequence of highly coordinated, genetically programmed morphogenetic events that involve directed growth and expansion of the facial prominences, programmed cell death, active fusion and then subsequent breakdown of the epithelial seam between the maxillary, medial nasal and lateral nasal processes. Even subtle abnormalities in any one of these events may lead to abnormalities, such as CL/P phenotype.

2.3 Abnormal lip development

Failure to coordinate the correct growth of the prominences leads to abnormal lip development. In this examination of the existing literature, I have concentrated on the two major abnormalities that can affect normal lip development: CL/P and FAS.

2.3.1 Cleft lip and/or palate (CL/P)

CL/P is the most common craniofacial disorder worldwide, with prevalence ranging from 1:550 in Asian and American Indian populations, 1:750 in Caucasians and 1:1,000 in African populations (Mossey, P.A. *et al*, 2009, Klotz, C.M. *et al*, 2010). Males are also more commonly affected by a ratio of 3:2 (whilst CPO is more common amongst females). The left side is more affected than the right side.

CL/P results in an array of complications affecting feeding, speech, hearing and psychological development (Mossey, P.A. *et al*, 2009). Patients undergo a series of surgical repairs starting from 3 months, and continuing until adulthood. They often require extensive dental and orthodontic treatment, speech and hearing therapy as well as psychological assessments and genetic counselling (Sitzman, T.J. *et al*, 2015).

Cleft phenotypes are highly variable, and can be classified according to several systems (Kernahan, D.A. *et al*, 1958, Mcbride, W.A. *et al*, 2016, Allori, A.C. *et al*, 2017). In the UK, the most widely used is the LAHSHAL system (Kriens, O., 1991), which describes the extent of the cleft, and also indicates whether it is unilateral or bilateral.

CL/P (which encompasses (CL) and (CLP)) is often considered a separate entity to cleft palate only (CPO) (Fraser, F.C., 1955). Often CL and CLP are grouped together in CL/P genetic studies under the assumption that CLP arises as a more severe form of CL (Dixon, M.J. *et al*, 2011). However, epidemiological studies suggest that CL may also arise as an independent entity to CLP (Harville, E.W. *et al*, 2005, Grosen, D. *et al*, 2010b).

2.3.1.1 Aetiology of CL/P

A CL occurs when there is failure of fusion between the maxillary process and the medial nasal process (Ohbayashi, N. *et al*, 1986). The palate may or may not also display a cleft. The aetiology of CL/P is complex encompassing both genetic (Tables 2.2 and 2.3) and environmental interactions (Table 2.4) (Cobourne, M.T., 2004). CL/P can also be divided according to two main subtypes: nonsyndromic (NSCL/P) and

syndromic (SCL/P). The exact mechanism of CL/P is poorly understood, and much of the understanding is based on mutant mice studies; two main theories have been hypothesised:

2.3.1.1.1 Failure of mesoderm migration

Failure of the mesoderm to proliferate could prevent the prominence from growing adequately in order to meet the opposing process. Teratogens may inhibit neural crest migration (Ashique, A.M. *et al*, 2002).

2.3.1.1.2 Failure of fusion

Surgical removal of the distal part of the medial nasal process resulted in a cleft in rat embryos. Whilst surgical removal of the lateral nasal process or the maxillary process did not prevent fusion of the other process with the medial nasal process (Ohbayashi N., E.K., 1986). From this it is concluded that contact and fusion of the maxillary and medial nasal process is not dependent on prior fusion between the lateral and medial nasal process.

Epithelial filopodia are also seen to establish bridges between the facial processes (Millicovsky P.J, J.M.C., 1981, Hinrichsen, K., 1985, Senders Cw, P.E., Hendrickx Ag, Cukierski Ma, 2003, Cox, T., 2004). These attachments are greatly reduced in A/WySn and CL/Fr mouse embryos, both of which exhibit a high frequency of spontaneous CL/P (Millicovsky P.J, J.M.C., 1981). Numerous mouse studies have also shown the significance of Tgf- β type1 receptor Alk5 for normal lip development and upper lip fusion. (Wai-Yee Li, M.D., Vesa Kaartinen, 2008).

2.3.1.2 Genetics of CL/P

The process of lip development is highly complex, and is mediated by a number of specific genetic signals and pathways. An alteration in any single signal could result in in a CL/P. Many genes have been implicated in the aetiology of both SCL/P and NSCL/P.

2.3.1.2.1 Syndromic cleft lip and/or palate (SCL/P)

Individuals with SCL/P display additional characteristic features and exhibit high familial aggregation rates (Farrall, M. *et al*, 1992, Dixon, M.J. *et al*, 2011). Several genes have been implicated in SCL/P, with over 300 Mendelian syndromes in humans including CLP as part of the phenotype (Gorlin Rj, C., Hennekam Rcm, 2001). Mendelian modes of inheritance are attributed to a single gene mutation (Dixon, M.J.

et al, 2011) and are highly penetrant. Many of the phenotype/genotype associations are attributed to linkage analysis studies of pedigree families (Table 2.2).

The genes identified so far include *PVRL1* in CLP-ectodermal dysplasia syndrome (CLPED1), which was identified in a group of individuals on Margarita Island, who have a much higher prevalence of CLPED1 compared with the general population (Sozen, M.A. *et al*, 2001). PVRL1 is a cell adhesion molecule, important during facial and palatal development (Sozen, M.A. *et al*, 2001).

TP63/P63 has been associated with ectrodactyly with ectodermal dysplasia and CLP (EEC) (Leoyklang, P. *et al*, 2006). Missense mutations in different parts of TP63 can give rise to different presentations of CL/P. TP63 has also been associated with NSCL/P (Leoyklang, P. *et al*, 2006) and may influence facial morphology (Liu, F. *et al*, 2012c).

IRF6 has been associated robustly with Van der Woude syndrome (Kondo, S. *et al*, 2002, Cobourne, M.T., 2004, Cox, T., 2004). In the mouse, *Irf6* expression is restricted to the palatal mesenchyme immediately prior to and during fusion, it may also interact with the expression of *Tgfb3* (Stanier, P. *et al*, 2012). IRF6 has also been associated with NSCL/P in some GWAS studies (Beaty, T.H. *et al*, 2010, Mangold, E. *et al*, 2011, Beaty, T.H. *et al*, 2013)

Mutations in *FGFR1* can result in Kallmann syndrome (Dode, C. *et al*, 2003), during normal upper lip development, *Fgf* signaling plays essential roles in midfacial growth, and normal variations could influence philtrum length and mouth width (Claes, P. *et al*, 2014).

FOXC2 mutations, which codes for a forkhead transcription factor involved in the development of the lymphatic and vascular system, can lead to Hereditary lymphedema-distichiasis and CP (Fang, J. *et al*, 2000, Yildirim-Toruner, C. *et al*, 2004. FOX genes are mediators of the Hh pathway during craniofacial development, and are necessary for cell survival and proliferation {Jeong, 2004 #569).

Mutations in the *SATB2* region are associated with glass syndrome, which is characterized by CP, micrognathia and dysmorphic facial features (Brewer, C.M. et al, 1999). *SATB2* directly interacts with the activity of transcription factors that regulate craniofacial development and also has a role in osteoblast differentiation and skeletal development in mice (Brewer, C.M. *et al*, 1999). The *Satb2* knockout mouse embryos had multiple craniofacial defects, including a significant truncation of the mandible, a

shortening of the nasal and maxillary bones, malformations of the hyoid bone and a CP (Dobreva, G. et al, 2006).

Cadherins play an essential role in neural crest development and migration (Cordero, D.R. *et al*, 2011), mutations in *E-cadherin* (*CDH1*) were found in two families with hereditary diffuse gastric cancer associated with CLP (Frebourg, T. *et al*, 2006).

Mutations in *EFNB1* are associated with craniofrontonasal syndrome (CFNS) (Twigg, S.R. *et al*, 2004), and individuals also have several phenotypes, including bifid nose and columellar indentation (Van Den Elzen, M.E. *et al*, 2014).

Furthermore, these genes also demonstrate overlap between SCL/P and NSCL/P (Stanier, P. *et al*, 2004).

Table 2.2 Candidate genes for SCL/P identified by conventional methods

Chromosomal locus	Gene	Syndrome	Phenotype MIM	Reference
1p21	*COL11A1*	Stickler Type 2	604841	(Francomano, C.A. *et al*, 1987) (Snead, M.P. *et al*, 1996)
1p31.1-33	*DHCR24*	Desmosterolosis (AR)	602398	(Waterham, H.R. *et al*, 2001)
1p36.3	*MTHFR*	OFC1	607093	(Mostowska, A. *et al*, 2006)
1q32.2-41	*IRF6*	Van der Woude	119300	(Kondo, S. *et al*, 2002)
2p13	*TGFα*	OFC2	190170	(Chenevix-Trench, G. *et al*, 1992, Shiang, R. *et al*, 1993)
2p21	*SIX3*	Holoprosencephaly	142945	(Wallis, D.E. *et al*, 1999)
2q14.2	*GLI2*	Holoprosencephaly	610829	(Roessler, E. *et al*, 2003)
2q24.1-33	*GAD67*	Pierre-Robin sequence	311895	(Jakobsen, L.P. *et al*, 2006)Jakobsen, L.P. *et al*, 2006)
2q32-q33	*SATB2*	Glass	612313	(Fitzpatrick, D.R. *et al*, 2003, Vieira, A.R. *et al*, 2005)
2q33.1	*SUMO1*	OFC10	601912	(Alkuraya, F.S. *et al*, 2006)
2q37.1	*CHRNG*	Lethal and escobar multiple pterygium	265000	(Morgan, N.V. *et al*, 2006)
3p14.3	*FLNB*	Larsen syndrome; atelosteogenesis	150250	(Krakow, D. *et al*, 2004)
3q28	*TP63*	Hay-Wells syndrome (AEC/EEC) (AD)	106260	(Garcia Bartels, N. *et al*, 2007)
4p16.2	*MSX1*	Hypodontia	142983	(Jezewski, P.A. *et al*, 2003, Suzuki, Y. *et al*, 2004)
5p13.1	*NIPBL*	Cornelia de Lange (AD)	122470	(Krantz, I.D. *et al*, 2004)
5p13.2	*C5orf42*	Oro-facial-digital	277170	(Lopez, E. *et al*, 2014)
5q32	*SLC26A2*	Diastrophic dysplasia (AR)	222600	(Hastbacka, J. *et al*, 1994)
5q32-33.1	*TCOF1*	Treacher Collins (AD)	154500	T.C.S., C.G. (1996)
6p21.3	*COL11A2*	Stickler Type 2	184840	(Brunner, H.G. *et al*, 1994)
6p24.3	*OFC1*	OFC1	119530	(Carinci, F. *et al*, 2007)
6p24.3	*TFAP2A*	Branchio-oculo-facial (AD)	113620	(Milunsky, J.M. *et al*, 2008)
7p21-p22	*TWIST1*	Saethre-Chotzen (AD)	101400	(Howard, T.D. *et al*, 1997)
7p22	*ACTB*	AD developmental malformations	607371	(Verloes, A. *et al*, 2015)
7q11.2-q21.3	*EEC1*	Ectrodactyly Ectodermal dysplasia	129900	Leoyklang et al, 2006
7q21.11	*CHD7*	CHARGE	214800	(Vissers, L.E. *et al*, 2004)
7q36	*SHH*	Holoprosencephaly	610829	(Roessler, E. *et al*, 2003, Vieira, A.R. *et al*, 2005)
8p12	*FGFR1*	Kallmann	136350	(Dode, C. *et al*, 2003)
8p21.1	*ESCO2*	Roberts Syndrome	268300	(Vega, H. *et al*, 2005)
9q22	*FOXE1*	Bamforth-Lazarus	602617	(Venza, I. *et al*, 2011a)
9q22.33	*TGFBR1/2*	Loeys-Dietz	609192	(Loeys, B.L. *et al*, 2005, Vieira, A.R. *et al*, 2005)
9q22.32	*PTCH1*	Holoprosencephaly / Gorlin-Goltz (AD)	109400	(Larsen, A.K. *et al*, 2014)
10q26.13	*FGFR2*	Crouzon/Apert/Pfeiffer	101200	(Wilkie, A.O. *et al*, 1995)
11q13.4	*DHCR7*	Smith-Lemli-Opitz (AR)	270400	(Waterham, H.R. *et al*, 2012)
11q23.3	*PVRL1 CLPED1*	Margerita Island Ectodermal Dysplasia	600644	(Sozen, M.A. *et al*, 2001)
11q24.2	*HYLS1*	Hydrocephalus	236680	(Mee, L. *et al*, 2005)
12q13.11	*COL2A1*	Stickler Type 1	609508	(Francomano, C.A. *et al*, 1987)
12q13.12	*MLL2*	Kabuki (AD)	147920	(Ng, S.B. *et al*, 2010)
13q33.1-q34	*TP73L*	OFC9	610361	(Carinci, F. *et al*, 2007)
14q22.2	*BMP4*	OFC11	600625	(Castilla, E.E. *et al*, 1995, Suzuki, S. *et al*, 2009)
16q22.1	*CDH1*	Familial gastric cancer and CL/P	192090	(Frebourg, T. *et al*, 2006)
16q22	*DHODH*	Postaxial Acrofacial dysostosis (POADS)	263750	(Fang, J. *et al*, 2012)
16q24.1	*FOXC2*	Hereditary lymphedema-distichiasis (AD)	602402	(Fang, J. *et al*, 2000, Yildirim-Toruner, C. *et al*, 2004)
17q21	*WNT3*	Tetra-amelia CLP	273395	(Niemann, S., 1993)
17q21.1	*RARα*		180240	(Chenevix-Trench, G. *et al*, 1992)
17q21-24.3	*SOX9*	Pierre-Robin sequence +/- Campomelic dysplasia (Y-Chr)	261800	(Foster, J.W. *et al*, 1994, Jakobsen, L.P. *et al*, 2006)Jakobsen, L.P. *et al*, 2006)
17q31.1-24.2	*KCNJ2*	Andersen (AD)	170390	(Andelfinger, G. *et al*, 2002)
18p11.3	*TGIF*	Holoprosencephaly	142946	(Gripp, K.W. *et al*, 2000)
19q13.2	*SIX5*	Branchiootorenal type 2	600757	(Rival, J.M. *et al*, 2001, Carinci, F. *et al*, 2007)
22q11deletion	*TBX1*	DiGeorge	188400	(De La Chapelle, A. *et al*, 1981, Kitsiou-Tzeli, S. *et al*, 2004, Vieira, A.R. *et al*, 2005)
Xp11.22	*PHF8*	Siderius x - linked mental retardation	300263	(Laumonnier, F. *et al*, 2005)
Xp11.22	*PQBP1*	X-linked mental retardation (MRX / MRXS) (X-Chr)	309530	(Kalscheuer, V.M. *et al*, 2003)
Xp11.4	*BCOR*	Oculofaciocardiodental	300166	(Ng, D. *et al*, 2004)
Xp22	*MID1*	Opitz GBBB	300000	(Quaderi, N.A. *et al*, 1997)
Xp22	*OFD1*	Oro-facial-digital T1	311200	(Ferrante, M.I. *et al*, 2001)
Xq13.1	*EFNB1*	Craniofrontonasal (X-Chr)	304110	(Twigg, S.R. *et al*, 2004)
Xq21.1	*TBX22*	CP+/- ankyloglossia, Abruzzo-Erickson	303400	(Braybrook, C. *et al*, 2001)
Xq28	*FLNA*	Otopalatodigital types 1 and 2	304120	(Robertson, S.P. *et al*, 2003)

2.3.1.2.2 Non-syndromic cleft lip and/or palate (NSCL/P)

The majority of CL/P cases (70%) are NSCL/P and have no other abnormalities (Beaty, T.H. *et al*, 2013). They arise sporadically and have low penetrance, with modest recurrence rates (Dixon, M.J. *et al*, 2011). The evidence that NSCL/P arise due to a genetic aetiology comes from studying the relative risk amongst family members and twin studies (Mossey, P.A. *et al*, 2009). Monozygotic twins have a 25%-60% increased occurrence of CL/P, whilst dizygotic twins have a rate of 3 - 10%, which is approximately the same as the sibling risk (Mitchell, L.E. *et al*, 1992, Christensen, K. *et al*, 1993).

The relative risk of having an offspring with CL/P from an affected or an unaffected twin is around 10 times higher compared to the background population (Grosen, D. *et al*, 2010a). Thus, given that OFC has a genetic aetiology, and monozygotic twins share 100% of their DNA, it would seem sensible to expect that both twins would be carrying susceptible genes, but a further (environmental) incident may also be necessary to form the OFC.

The variability in the presentation of NSCL/P amongst family members, lead to the hypothesis that genes could also contribute to the incidence of NSCL/P (Cox, T., 2004). Linkage analysis studies have failed to identify these, however, numerous genetic precursors have been proposed through GWAS (Table 2.3). Although, it is widely accepted that there may still be many more, which are as yet, undiscovered (Beaty, T.H. *et al*, 2013).

Genome-wide association analysis of case-parent trios has been used to assign genetic associations with NSCL/P (Table 2.3). Studies have demonstrated associations with *IRF6, ABCA4* and *MAFB*, NOG and the 8q24 region (Birnbaum, S. *et al*, 2009, Mangold, E. *et al*, 2009, Mangold, E. *et al*, 2010, Beaty, T.H. *et al*, 2010)

ABCA4 (ATP-binding cassette protein A4) encodes an ATP-binding cassette transporter, and has been associated with Stargardt's disease, which is an autosomal recessive retinal degenerative disease. *Abca4* expression has not been observed in mouse palatal shelves around the time of palatal fusion (Beaty, T.H. *et al*, 2010), however, the peak SNP occurred in an intron of *ABCA4* (Beaty, T.H. *et al*, 2013). The intronic region does contain a transcriptional enhancer in the homologous mouse region, which is active in craniofacial tissues during embryonic development (Attanasio, C. *et al*, 2013).

Possibly, the human region may also contain a craniofacial enhancer (Beaty, T.H. *et al*, 2013).

MAFB (v-maf musculoaponeurotic fibrosarcoma oncogene homolog B) encodes a basic leucine zipper transcription factor. Either side of it are numerous binding sites for transcription factors known to play a role in palate development (including *MSX, IRF, SOX and BACH* families) (Dixon, M.J. *et al*, 2011). In the mouse, *Mafb* is highly expressed in the epithelium during palatal fusion (Beaty, T.H. *et al*, 2010).

A 640-kb noncoding interval at 8q24 has been robustly associated with NSCLP (Birnbaum, S. *et al*, 2009, Beaty, T.H. *et al*, 2010, Beaty, T.H. *et al*, 2013). This interval has been shown to contain remote cis-acting enhancers that control *Myc* expression in the developing face, and deletion leads to misexpression of several downstream genes, which results in mild alteration of facial morphology and sporadically to CL/P in mice (Uslu, V.V. *et al*, 2014).

A subsequent meta-analysis revealed *PAX7, COL8A1/FILIP1L* and *NTN1* achieved genome-wide significance (Ludwig, K.U. *et al*, 2012, Beaty, T.H. *et al*, 2013).

PAX7 (paired box 7) is a member of the paired box family of transcription factors; and they control epithelial-mesenchymal transitions, differentiation and proliferation (Francis-West, P.H. *et al*, 2003). Inhibition of *Pax7* protein in mice prevented the expression of several NCC markers, including *Slug (Snail2), Sox9, Sox10 and HNK-1* (Blake, J.A. *et al*, 2014).

COL8A1 encodes one of the two alpha chains of type VIII collagen and is expressed by corneal and vascular endothelial cells. Knockout mice have demonstrated that *COL8A1* plays an important role in normal eye development (Hopfer, U. *et al*, 2005).

NTN1 is thought to be involved in axon guidance and cell migration during development (Charron, F. *et al*, 2005); it is a chemo attractant, which draws axons towards the midline during development (Kennedy, T.E. *et al*, 1994).

Near hits were also found on *THADA, DCAF4L2, GADD45G, RBFOX3* and *FOXE1* {Beaty, 2013 #234}(Ludwig, K.U. *et al*, 2014).

THADA (Thyroid adenoma associated) is likely to be involved in the death receptor pathway and apoptosis and are associated with benign adenomas of the thyroid (Rippe, V. *et al*, 2003).

DCAF4L, is a scaffolding protein required for craniofacial development (Leslie, E.J. *et al*, 2016) and elevated expression has been associated with colorectal cancer (Wang, H. *et al*, 2016).

GADD45G is a member of a group of genes whose transcript levels are increased following stressful growth arrest conditions and treatment with DNA-damaging agents (Zhan, Q. *et al*, 1993).

RBFOX3 mutations are linked to epilepsy and cognitive impairments (Wang, H.Y. *et al*, 2015).

FOXE1 belongs to the forkhead family of transcription factors (Venza, I. *et al*, 2011b), it functions as a thyroid transcription factor that plays a role in thyroid morphogenesis. Mutations are associated with the Bamforth-Lazarus syndrome, and with susceptibility to nonmedullary thyroid cancer-4 (Carre, A. *et al*, 2014).

In addition, *ABCA4, PAX7, IRF6, 8q24, NTN1, NOG, and MAFB* have achieved subsequent replication in further population samples (Table 2.3). Many of these genes have been previously implicated in SCL/P, suggesting that genetic variants may also give rise to normal variation, and not necessarily, a full cleft.

Table 2.3 Candidate genes for NSCL/P identified by GWAS

SNP	Locus	Nearby gene	NSCL/P genetic allele	GWAS P-Values
rs560426	1p22.1 - 1p21.3	*ABCA4 **	G	$5.01x10^{-12}$ (Beaty, T.H. *et al*, 2010) $3.14x10^{-12}$ (Ludwig, K.U. *et al*, 2012)
rs742071	1p36 - 1p36.13	*PAX7 **	T	$7.02x10^{-9}$ (Ludwig, K.U. *et al*, 2012) $1.59x10^{-6}$ (Beaty, T.H. *et al*, 2013)
rs861020	1q32.2 - 1q41	*IRF6 **	A	$1.20x10^{-9}$ (Beaty, T.H. *et al*, 2010) $3.24x10^{-12}$ (Ludwig, K.U. *et al*, 2012)
rs7590268	2p21	*THADA**	G	$1.25x10^{-8}$ (Ludwig, K.U. *et al*, 2012) $1.49x10^{-3}$ (Beaty, T.H. *et al*, 2013)
rs7632427	3p11.1	*EPHA3*	T	$3.90x10^{-8}$ (Ludwig, K.U. *et al*, 2012)
rs793464	3q12.1	*COL8A1/ FILIP1L*	A	$4.49x10^{-5}$ (Beaty, T.H. *et al*, 2013)
rs12543318	8q21.3	*DCAF4L2*	C	$1.90x10^{-8}$ (Ludwig, K.U. *et al*, 2012) $7.68x10^{-6}$ (Beaty, T.H. *et al*, 2013)
rs987525	8q24*	*MYC*	A	$3.34x10^{-24}$ (Birnbaum, S. *et al*, 2009) $9.18x10^{-8}$ (Grant, S.F. *et al*, 2009) $1.11x10^{-16}$ (Beaty, T.H. *et al*, 2010) $5.12x10^{-35}$ (Ludwig, K.U. *et al*, 2012)
rs1007966	9q22.1 - 9q22.2	*GADD45G*	G	$3.01x10^{-5}$ (Beaty, T.H. *et al*, 2013)
rs6478391	9q22.33	*FOXE1*	C	$6.83x10^{-3}$ (Beaty, T.H. *et al*, 2013)
rs7078160	10q25	*VAX1*	C	$1.92x10^{-8}$ (Mangold, E. *et al*, 2010) $3.96x10^{-11}$ (Ludwig, K.U. *et al*, 2012)
rs8001641	13q31.1	*SPRY2*	A	$2.62x10^{-10}$ (Ludwig, K.U. *et al*, 2012)
rs1873147	15q22.2	*TPM1*	C	$2.23x10^{-5}$ (Beaty, T.H. *et al*, 2013)
rs1880646	17p13.1	*NTN1 **	C	$2.81x10^{-8}$ (Ludwig, K.U. *et al*, 2012)
rs227731	17q22	*NOG **	C	$1.07x10^{-8}$ (Mangold, E. *et al*, 2010) $1.78x10^{-8}$ (Ludwig, K.U. *et al*, 2012)
rs2612753	17q25.3	*RBFOX3*	T	$3.29x10^{-4}$ (Beaty, T.H. *et al*, 2013)
rs13041247	20q12	*MAFB*	T	$1.44x10^{-11}$ (Beaty, T.H. *et al*, 2010) $6.17x10^{-9}$ (Ludwig, K.U. *et al*, 2012)

Candidate genes for NSCL/P identified by genome-wide association studies.
"*" Denotes variants that have also been replicated in independent studies, and hence represent robust associations

2.3.1.2.3 Sub clinical cleft phenotypes

Subclinical phenotypes of CL/P, including discontinuities in the orbicularis oris muscle (Weinberg, S.M. *et al*, 2008a), lip pits/whorls (Neiswanger, K. *et al*, 2009) and increased upper facial width (Weinberg, S.M. *et al*, 2009, Weinberg, S.M. *et al*, 2008b) have been observed amongst unaffected family members. However, no universal classification system exists which accurately records subclinical phenotypes (Mcbride, W.A. *et al*, 2016).

There has been some interest in assessing the underlying musculature of the lips for defects. A histological study of a cadaver with a unilateral CLP demonstrated chaotic orbicularis oris muscle fibres, with indistinguishable muscle layers (Kernahan, D.A. *et al*, 1984). Ultrasonography has been suggested at a tool for non-invasively assessing the orbicularis oris muscle for defects (Rogers, C.R. *et al*, 2009). Discontinuities of the orbicularis oris muscle have been observed amongst 10.3% of unaffected family members of CL/P, compared with 5.8% of controls, giving an odds ratio of 1.74 ($P < 0.01$) (Klotz, C.M. *et al*, 2010). As a result of this, some authors have postulated that it may be a sub-clinical phenotype of NSCL/P (Neiswanger, K. *et al*, 2007, Klotz, C.M. *et al*, 2010). However, other studies have not achieved significant findings (Leslie, E.J. *et al*, 2016).

The most common form of SCL/P is Van der Woude syndrome, and it is characterised in 85% of cases by the addition of lower lip pits. Lip pits or whorls have an increased prevalence amongst unaffected family members of Van der Woude (Neiswanger, K. *et al*, 2009), and have also been observed in cases of NSCL/P (Beaty, T.H. *et al*, 2013).

An increased upper facial width, including an increased mouth width has been suggested to contribute to craniofacial shape differences of unaffected relatives (Yoon, Y.J. *et al*, 2004, Weinberg, S.M. *et al*, 2008b). In addition, authors have suggested different risk features amongst mothers and fathers (Yoon, Y.J. *et al*, 2004, Weinberg, S.M. *et al*, 2009). Mothers of unaffected relatives have demonstrated increased upper facial width (Weinberg, S.M. *et al*, 2009), whilst males had increased cranial base width, increased lower facial height and decreased upper facial height compared with controls (Weinberg, S.M. *et al*, 2009), proposing that the phenotypes are partly sex-specific (Weinberg, S.M. *et al*, 2009).

2.3.1.3 Environmental influences of CL/P

Many environmental risk factors have been implicated (Table 2.4) through epidemiological and experimental data (Mossey, P.A. *et al*, 2009). A meta-analysis of 24 case-control and cohort studies demonstrated a statistically significant association between CL/P and tobacco smoking (Little, J. *et al*, 2004), indicating a relative risk of 1.34 (95%CI:1.25-1.44). Another study proposed an interaction between maternal smoking and genes *GRID2* and *ELAVL2*; however, it failed to gain statistical significance (Beaty, T.H. *et al*, 2013). This may actually be underestimated if one considers the risk associated with passive smoking. The teratogenic effect of smoking and alcohol has also been observed with a genetic variant of *Tgfα* (Romitti, P.A. *et al*, 2007).

The other environmental factors listed (Table 2.4) are associated with less convincing evidence, with inconsistent results. This could be attributed to the study design, which is often cohort or case-control studies, which could not account for confounders. It would be unethical to perform a randomised control trial to test these hypotheses.

Folic acid deficiency has been previously implicated with neural birth defects, and many authors have postulated its role in cleft aetiology, however, there is still unconvincing evidence that this is protective in cleft prevention (Gildestad, T. *et al*, 2015).

Maternal viral infection may also increase the risk of orofacial clefts (OFC), one theory is that it may induce maternal hyperthermia; another is that it may activate interferon regulatory transcription factors (e.g. *IRF6*) (Botto, L.D. *et al*, 2002).

Table 2.4 Environmental factors associated with CL/P

Maternal effect	Environmental incident
Maternal hypoxia	Maternal smoking (Honein, M.A. *et al*, 2007), alcohol abuse (Romitti, P.A. *et al*, 2007), drug abuse
Maternal health	Viral infection leading to maternal hyperthermia and malnutrition (Botto, L.D. *et al*, 2002)
Maternal diet	Excess Vitamin A (Mitchell, L.E. *et al*, 2003), Folic acid deficiency
Maternal Medication	Anticonvulsants (Dravet, C. *et al*, 1992), hypertensives, corticosteroids (Park-Wyllie, L. *et al*, 2000)
Others	Nitrate compounds, organic solvents, lead exposure, Pesticide exposure (Garcia, A.M., 1998)

2.3.2 Foetal Alcohol Syndrome (FAS)

FAS is a continuum of birth defects that results in mental and physical developmental delay, as well as reported characteristic facial features, comprising a smooth philtrum, thin upper lip vermilion and short palpebral fissure length.

The reported incidence of FAS varies worldwide. This could be attributed to the difficulty in diagnosing FAS due to the variable expressivity of the disorder, or the varying levels of alcohol consumption amongst different population groups. It is also suggestive of a genetic predisposition to the toxicity of alcohol amongst some populations. In the UK, there is no reported prevalence, however there is a universal estimate of around 1 in 1,000 (Abel, E.L., 1995) The figure varies amongst other studies from 4.6 in 1,000 in some parts of the USA to 1.3 in France (Sampson, P.D. *et al*, 1997).

Diagnosis is difficult, as its features cover a broad range in severity ranking. It requires a known history of alcohol ingestion during pregnancy and characteristic facial features. However, these facial features do not always occur in unison, and can occur in isolation. The Likert scale (Figure 1.12) was developed as an aid to the diagnosis of the lip phenotypes associated with this condition (Astley, S.J. *et al*, 1995).

The aetiology of FAS is thought to be multifactorial; encompassing a genetic predisposition (Ahlgren, S.C. *et al*, 2002) as well as maternal alcohol consumption (although the levels of alcohol required to cause damage is largely unknown). The current guidelines for the safe consumption of alcohol during pregnancy suggest that alcohol should be avoided altogether (Officer, C.M., 2015).

A recent study (Suttie, M. *et al*, 2013) of 3D scans of unaffected and affected individuals from South Africa, attempted to group individuals who were known to have been heavily exposed to alcohol prenatally, but did not show the characteristic facial phenotype. Dense surface modelling techniques and signature analyses were employed to determine agreement between clinical categorisation and the classifications induced from the face shape alone, to visualise facial differences and consider predictive links. They concluded that alcohol consumption might also influence additional subclinical features; however, more work would need to be carried out in this area to recognise what exactly these might be.

2.4 Phenotype/genotype associations

Traditionally, genetic-phenotype associations have been made according to the theory of Mendelian inheritance. This is based on the principle that both parents hold a set of two possible alleles. During the process of sexual reproduction, each gamete (egg/sperm) will contain either one of two possible alleles. For example, a (recessive allele) or A (dominant allele), if both parents contain combinations aA, this can give rise to four possible patterns: 25% aa, 50% aA or 25% AA. Therefore, if A is the dominant trait, there is a 25% chance that the offspring won't display the trait (and also won't pass on the trait), and a 75% chance that the trait will be expressed.

Mendelian inheritance models suggest that one gene affects one feature, and in many syndromic conditions, this may well be the case. However, with regards to normal facial variation, this model is too simplistic, with the likelihood that many genes may control a group of features (Liu, F. *et al*, 2012b).

2.4.1 Genetic mediators

It is well recognised that the human face is highly heritable; twin studies have demonstrated that nearly 80% of facial features are genetically inherited (Liu, F. *et al*, 2012a, Peng, S. *et al*, 2013), the rest is thought to be down to environmental incidents. Numerous genes and gene pathways have been identified as critical for craniofacial development. However, little is known as to which genes affect facial variation. The genetic effects of facial variation has mainly arisen from studies of congenital abnormalities or syndromes, but more recently genome-wide association techniques have made successful associations.

2.4.2 Gene association study designs

Linkage analysis (LA) and genome wide association study (GWAS) are the two analytical methods for mapping genes against human traits. Linkage study designs are limited to assessing phenotypes amongst pedigree families, and have therefore been useful in identifying genes associated with syndromes. GWAS methods search the entire genome for genetic associations of a particular disease, in a non-hypothesis-driven manner. They are deemed superior to LA, as they provide greater power and resolution (Risch, N. *et al*, 1996).

2.4.2.1 Genome-wide association study (GWAS)

GWAS usually follow the format of case-control studies, whereby participants' entire genomes are scanned for markers of genetic variation, without any prior knowledge about the location of potential susceptible alleles. Using this method, GWAS aims to identify genetic associations with observable traits. If the genetic variations are more frequent in the “cases”, it can be concluded that they are "associated" with the disease, and may work as pointers to the region of the human genome where the disease-causing problem is likely to reside. (Manolio, T.A. *et al*, 2009). For this reason, it can be used when the phenotype has a much lower penetrance, although it requires large study samples in order to observe the effect.

Genome association methods are only possible due to the phenomenon of haplotypes and genetic linkage. Haplotypes are a group of genes or a cluster of single nucleotide polymorphisms (SNPs) that are inherited together. SNPs can be used as markers to identify variations in the allele frequency at single positions in the DNA sequence amongst individuals. These groups of genes would have been inherited together from a single parent, either from a single chromosome or several chromosomes because of genetic linkage.

By examining haplotypes, scientists can identify patterns of genetic variation that are associated with health and disease states. For instance, if a haplotype is associated with a certain disease, then it is leads one to examine stretches of DNA near the SNP cluster to try to identify the gene or genes responsible for causing the disease.

Facial phenotypes, however, are only partially controlled by genes, a complex interaction between multiple genes and the environment occurs, which makes trying to understand and quantify human phenotypic variation difficult. Therefore, instead of looking at complex human traits, several researchers went straight to the source and looked for SNP sequences in the genome, in order to quantify individual human variation.

Individual variations are believed to arise due to SNPs and it has been reported that there may be as many as 150,482,731 SNPs in humans (Ncbi, 2015). It is estimated that 90% of human variation is observed as a result of <1% variation at 10 million sites (Kruglyak, L. *et al*, 2001). The remaining 10% is due to a vast array of variants that are rare in the population.

Most genetic variations are associated with the geographical and historical populations in which the mutations first arose. This ability of SNPs to tag surrounding blocks of ancient DNA (haplotypes) underlies the rationale for GWAS. However, in order to control for population stratification, studies must take account of the geographical and racial background of participants.

GWAS has allowed researchers to sample 500,000 or more SNPs from each subject in a study, capturing variation uniformly across the genome. To date, these studies have identified risk and protective factors for asthma, cancer, diabetes, heart disease, mental illness and other human differences (Bush, W.S. *et al*, 2012, Grant, S.F. *et al*, 2009). GWAS test the common disease/common variant hypothesis, whereby common diseases have multiple susceptibility alleles, each with small effect sizes (typically increasing disease risk between 1.2–2 times the population risk) (Bush, W.S. *et al*, 2012).

2.5 Genetics and facial morphology

Many syndromes have characteristic facial features, and often a clinical diagnosis is made based on particular features in addition to signs or symptoms of a medical condition. Certain observed associations of lip phenotypes with particular syndromes have been reported (Table 2.5).

Table 2.5 Characteristic lip phenotypes associated with syndromes

Feature	Syndrome	Gene	Locus
Narrow philtrum	Premature ageing syndrome	*PDGFRB*	5q32
Narrow & short philtrum	Cohen syndrome	*VPS13B*	*8q22.2*
	Floating-Harbor syndrome	*SRCAP*	16p11
	Wiedemann-Steiner Syndrome	*KMT2A*	11q23.3
	Foetal valproate syndrome		
	Miller-Dieker syndrome (Lissencephaly)	*LIS1*	17p13.3
	FAS (ADH1A)	*SHH*	4a23
	Geleophysic dysplasia	*ADAMTSL2*	9q34
	Ohdo syndrome (x-linked)	*MED12*	Xq13
	Geleophysic dysplasia 2	*FBN1*	15q21.1
	Kaufman oculocerebrofacial syndrome	*UBE3B*	12q23
Flat Cupid's bow shape and wide philtrum	Ackerman Syndrome		
	Pitt-Hopkins Syndrome	*TCF4*	18q21.2
Thin vermilion	Mandibulofacial dysostosis	*EFTUD2*	17q21.31

2.5.1 Candidate SNP studies and facial morphology

Research attempts have been made to determine how common NSCL/P SNPs may affect normal facial morphology (Boehringer, S. *et al*, 2011, Liu, F. *et al*, 2012b, Peng, S. *et al*, 2013, Miller, S.F. *et al*, 2014a). Study designs involve assessing for variations in morphological features between case and controls, and the methods have involved landmarking regions of the face, and subsequent analysis of distances, or PCA. Using this methodology, the majority of successes so far have involved regions attributable to bony landmarks, in particular those that have good reproducibility (Table 2.6). Unaffected siblings of NSCL/P have wider noses and bizygomatic distances compared with controls (Boehringer, S. *et al*, 2011).

Table 2.6 Facial phenotype/genotype associations arising from population studies

Locus	Gene	Phenotype	Method	Population	SNP	P-Values
1p31.3	*SLC35D1*	Periorbital region	Candidate SNPs 3D BRIM	592 Americas & West Africa	rs1074265	<0.0005 (Claes, P. *et al*, 2014)
1q32.2-1q41	*IRF6*	Lip prominence / thickness (females)	Candidate SNPs 3D high density registration	1001 (604 females, 397 males) Han Chinese	rs642961	$6x10^{-5}$ (Peng, S. *et al*, 2013)
1p36.23-1p36.33	*PRDM16*	Nasal width/nasal tip	GWAS 3D MRI/2D photos	5,388 Caucasian	rs4648379	$1.1x10^{-8}$ (Liu, F. *et al*, 2012a)
1q42.12	*LEFTY1*	Mid-face A-P projection	Candidate SNPs 3dMD PCA	188 Case, 194 Controls Caucasian	rs3766941	0.023 (Miller, S.F. *et al*, 2014a)
2q22.3	*ZEB2*	Chin dimple	GWAS self reported traits	5,388 Caucasian		$4x10^{-5}$ (Eriksson, N. *et al*, 2012)
2q35	*PAX3*	Nasal bridge prominence	GWAS 3D Landmarking	4,747 Caucasian	rs7559271	$4x10^{16}$ (Paternoster, L. *et al*, 2012)
			GWAS 3D MRI/2D photos	5,388 Caucasian	rs974448	(Liu, F. *et al*, 2012a)
3q28	*TP63*	Inter-pupillary distance	GWAS 3D MRI/2D photos	5,388 Caucasian	rs17447439	$4.4x10^{-8}$ (Liu, F. *et al*, 2012a)
5p12 – 5p13	*GHR*	Mandibular ramus length	Candidate SNPs & linear measurements LC	145 Chinese	rs6180	(Zhou, J. *et al*, 2005)
5q35.1	*C5orf50*	Nasion	GWAS 3D MRI/2D photos	5,388 Caucasian	rs6555969	$5.8x10^{-9}$ (Liu, F. *et al*, 2012a)
6q22-6q23	*ENPP1*	Upper facial height	Candidate SNPs & head sized traits	1042 Western Eurasian	rs7754561	$9x10^{-5}$ (Ermakov, S. *et al*, 2010)
8p21.23	*FGFR1*	Long philtrum, wide mouth	Candidate SNPs & 3dMD BRIM	592 Americas& West Africa	rs13267109	<0.0005 (Claes, P. *et al*, 2014)
8q24.21	*CCDC26*	Bizygomatic distance	Candidate SNPs 2D photos 3D MRI	Caucasian	rs987525	0.017 (Boehringer, S. *et al*, 2011)
10q24.3	*COL17A1*	Inter-pupillary distance	GWAS 3D MRI/2D photos	5,388 Caucasian	rs805722	$4.0x10^{-8}$ (Liu, F. *et al*, 2012a)
12p13.2	*LRP6*	Thick upper lip	Candidate SNPs 3dMD BRIM	592 Americas & West Africa	rs2724626	0.034 (Claes, P. *et al*, 2014)
15q13.3	*GREM1*	Nose Width	Candidate SNPs 2D photos 3D MRI	Caucasian	rs1258763	$6x10^{-4}$ (Boehringer, S. *et al*, 2011)
20q12	*MAFB*	AP position chin	Candidate SNPs 3dMD PCA	188 Case, 194 Controls Caucasian	rs11696257	0.004 (Miller, S.F. *et al*, 2014a)

2.6 Summary

Lip formation takes place during the early stages of embryonic development and is tightly regulated by complex signalling factors. Genetic variations or environmental injuries can affect the normal development of the lip.

It has been hypothesised that NSCL/P would best fit an oligogenic model, where one or a few major genes are influenced by a small number of environmental modifiers (Marazita, M.L. *et al*, 1986, Farrall, M. *et al*, 1992, Fitzpatrick, D. *et al*, 1993). In addition, a threshold model has also been hypothesised (Sivertsen, A. *et al*, 2008, Grosen, D. *et al*, 2010b), whereby a certain combination of genes or a particular environmental injury needs to occur, to tip the balance towards an OFC occurring.

There is a suggestion that disease NSCL/P genetic alleles may also modulate the phenotypes of unaffected carriers, within a range of normal variation. Subtle shape alteration patterns may help to screen carriers of disease alleles and therefore facilitate disease prevention. Identification of sub-clinical cleft phenotypes may be important in a clinical setting, as a means of providing more accurate recurrence risk estimates to families at risk of OFC.

Variation in craniofacial morphology often forms part of the diagnostic procedure for genetic disorders; however, few genetic variants that influence normal facial variation in the general population have been explored.

Chapter 3 Research aims and objectives

3.1 Research aims and objectives

The literature review has highlighted that there is much variation in individual lip morphology. However, the current methods of analysing lip phenotypes are limited to landmarking methods, or characterising a few shapes.

Whilst it is evident that the lips are under strong genetic control, little has been discovered as to how these genes affect normal variation of the lip phenotypes.

The overarching aim of this study was to investigate the biological basis of lip phenotypes

The objectives were to:

- Develop a robust, reproducible classification system of lip phenotypes
- Measure the prevalence of lip phenotypes within a population sample of 4,747
- Assess for associations of lip phenotypes with other lip phenotypes
- Assess if there were any sex variations of lip phenotypes
- Assess the reproducibility of the classification system with other acquisition methods
- Perform a genome-wide association study (GWAS) of lip phenotypes
- Assess for associations of non-syndromic cleft lip/palate (NSCL/P) single nucleotide polymorphs (SNPs) to lip phenotypes
- Perform a case/control study to assess the prevalence of lip phenotypes amongst unaffected biological parents of cleft and non-cleft children
- Assess the predictive capability of lip phenotypes as a precursor of cleft risk

Chapter 4: Development of a robust lip classification scale

4.1 Introduction

Chapter 1 explored the limitations of the current methods of phenotyping lip phenotypes. Locating landmarks were demonstrated to be advantageous, as they were accurate and reproducible (Toma, A.M. *et al*, 2009). Characterisation of lips is sparsely covered in the scientific literature (Table 1.4), and as such, many of the subtle features of the soft tissue variations of the lips have been overlooked. Characterising lip phenotypes can provide more information about lip detail and shape variations.

4.1.1 Objectives

The objectives were to:

- Develop a robust, reproducible classification system of lip phenotypes
- Measure the prevalence of lip phenotypes within a population sample of 4,747
- Assess for associations of lip phenotypes with other lip phenotypes
- Assess if there were any sex variations of lip phenotypes

4.2 Methodology

4.2.1 The Sample

The subjects of this study were recruited from ALSPAC, also known as Children of the 90's. ALSPAC recruited more than 14,000 pregnant women with estimated dates of delivery between April 1991 and December 1992. The vast majority of mothers (94.7%) and fathers (94.1%) reported themselves to be of white ethnicity. Less than 1% were of black (Caribbean/African/other) origin and less than 1% of Asian (Indian/Pakistani/Bangladeshi/Chinese) origin (Boyd, A. *et al*, 2013). The rest were either missing or other.

The demographic profile of the ALSPAC sample has an under-representation of non-White minority ethnic groups compared with the national population. This may influence external validity of some study findings based on prevalence, although it should not influence adversely the longitudinal results provided the features affecting bias are included (Boyd, A. *et al*, 2013).

The initial sample consisted of 14,541 pregnancies, this is the number of pregnancies for which the mother enrolled in the ALSPAC study and had either returned at least one questionnaire or attended a "Children in Focus" clinic by 19/07/99. Out of the initial 14,541 pregnancies, all but 69 had known birth outcomes. Of these 14,472 pregnancies, 195 were twins, 3 were triplets and 1 was a quadruplet pregnancy, resulting in 14,676 foetuses within the initial ALSPAC sample. Of these, 14,676 foetuses, 14,062 were live births and 13,988 were still living after one year. Individuals from the study have been followed up since birth, with detailed data collected throughout childhood (Golding, J. *et al*, 2001).

When the oldest children were approximately 7 years of age, an attempt was made to bolster the initial sample with eligible cases that failed to join the original study. As a result, when considering variables collected from the age of seven onwards (and potentially abstracted from obstetric notes), the data available was for more than the 14,541 pregnancies mentioned above. The number of new pregnancies not in the initial sample that are currently represented on the built files is 542, of which, 6 produced twins, meaning that the number of additional children was 548. The total sample size for analyses using child based questionnaire data collected after age seven was therefore 15,224, of which 14,610 were live births and 14,535 were living after one year.

A 10% sample of the ALSPAC cohort, known as the Children in Focus (CiF) group, attended clinics at the University of Bristol at various time intervals between 4 to 61 months of age. The CiF group was chosen at random from the last 6 months of ALSPAC births (1432 families attended at least one clinic). Excluded were those mothers who had moved out of the area or were lost to follow-up, and those partaking in another study of infant development in Avon. Exclusion criteria included subjects with craniofacial abnormalities and subjects with facial disfigurement.

4.2.1.1 Sample available for phenotyping

At age fifteen, 9,985 subjects were invited to take part in further clinics, of which 5,253 children attended. Of these 506 individuals were excluded as they either did not have facial images recorded, or the images were of poor quality, obvious ethnicity or facial dysmorphology (Boyd, A. *et al*, 2013).

Four thousand seven hundred and forty-seven children (2,514 females and 2,233 males) had 3D scanned images taken. Of these, there were 16 twin pairs and no triplets or quads. Around 10% of the subjects are related between 1st cousin and sibling.

Four thousand three hundred of the four thousand seven hundred and forty-seven mothers reported ethnicity of their children. Ninety-six percent (4,136) were white and four percent (164) were non-white.

Table 4.1 Distribution of the ALSPAC sample

	Data available
Total ALSPAC sample	14535
Invited to have facial scans	9985
Attended for facial scans	5253
Available for phenotyping	4747

4.2.2 Ethical approval

Ethical approval for this study was obtained from the Central & South Bristol Research Ethics Committee (UBHT): 06/Q2006/53 Avon Longitudinal Study of Parents and Children (ALSPAC), Hands on Assessments: Teen Focus 3 (Focus 15+). (7th August 2006) (Confirmed 15th September 2006)

Written consent was also obtained from parents and guardians prior to obtaining the facial scans.

4.2.3 Image Capture

The 3D images of the subjects were captured using two commercially available, high-resolution Konica Minolta Vivid VI900® cameras, with a reported manufacturing accuracy of 0.1mm. The details of capture are widely published (Kau, C.H. *et al*, 2005b, Kau, C.H. *et al*, 2008)

First, the images were cleaned automatically in a process that removed large unwanted areas in the scans. This was subsequently followed by the manual fine cleaning of the images, which involved the removal of minor interferences and the in-filling of small “holes” or voids in the images, whilst preserving the overall shape and contour of the face.

The raw images had a semi-rough texture due to the irregularity of the surface contours and background noise (reflection of light off the surfaces of the subject). The software used a smoothing process whilst preserving the overall shape and volume.

The left and right scans were then aligned to one another using the iterative closest point algorithm (ICP), based on the overlap of the two facial shells.

The 3D facial shells were then imported to Rapidform® 2006 software (INUS Technology Inc. Seoul, South Korea) and subsequently six images were exported in JPEG format; frontal, left and right side profiles, left and right three-quarters and submentovertex (SMV) view (Figure 4.2). This ensured the images were viewed in a standardised format, magnification and orientation. Images were viewed in greyscale (without texture), as it was found that these images produced the most reliable, consistent analyses and were not subject to significant texture variation due to body temperature or ambient room temperature.

4.2.4 Development of the classification scale

The scale was initially constructed from lip characterisation methods previously described in the literature review (Mori, A. *et al*, 2005, Carey, J.C. *et al*, 2009, Hennekam, R.C. *et al*, 2009). It consisted of 4 philtrum region phenotypes, two upper and lower lip phenotypes, commissures and three sub-lip phenotypes. Each phenotype consisted of a normal trait (middle column), a reduced/absent trait (left column) and an exaggerated trait (right column) (Figure 4.1).

A sample of twenty randomly selected subjects (Figure 4.3) was initially assessed, in order to identify lip phenotypes according to this rudimentary scale It soon became apparent that there were additional features present that had not been previously described, such as detailing the appearance of the inferior border of the upper lip (groove / drop), or the roped appearance to the vermilion borders (double vermilion border).

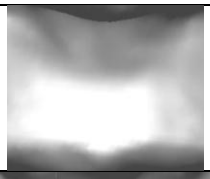
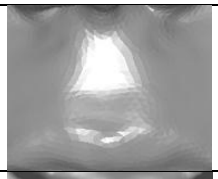
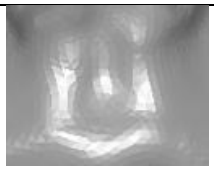
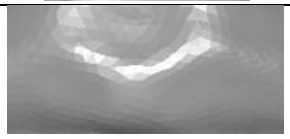
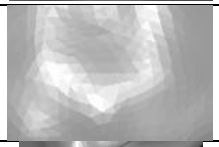
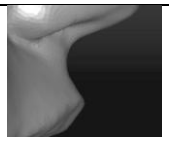
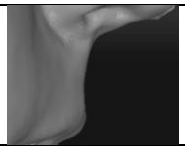
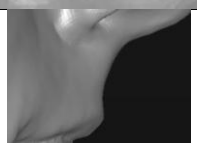
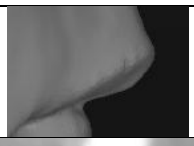
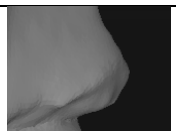
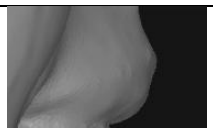
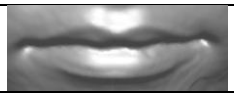
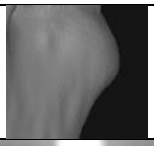
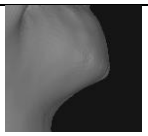
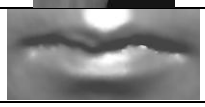
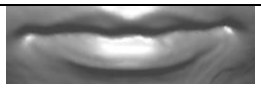
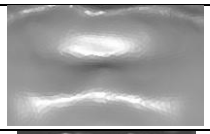
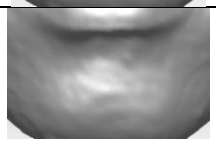
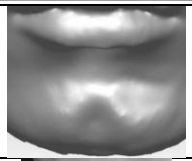
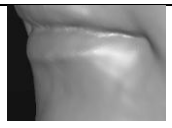
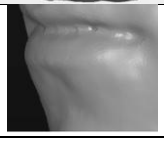
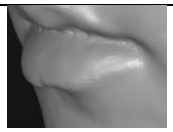
	0 (Reduced)	1 (Normal)	2 (Exaggerated)
Philtrum			
Philtrum Shape			
Philtrum Width			
Cupid's Bow Shape			
Nasolabial angle (Profile)			
Upper Vermilion			
Vermilion Fullness (Profile)			
Vermilion border			
Lower Vermilion			
Vermilion Fullness (Profile)			
Vermilion border			
Commissures			
Commissures			
Sub-Lip			
Mentolabial fold			
Chin dimple			
Lower lip protrusion			

Figure 4.1 Rudimentary classification scale for the characterisation of lip phenotypes

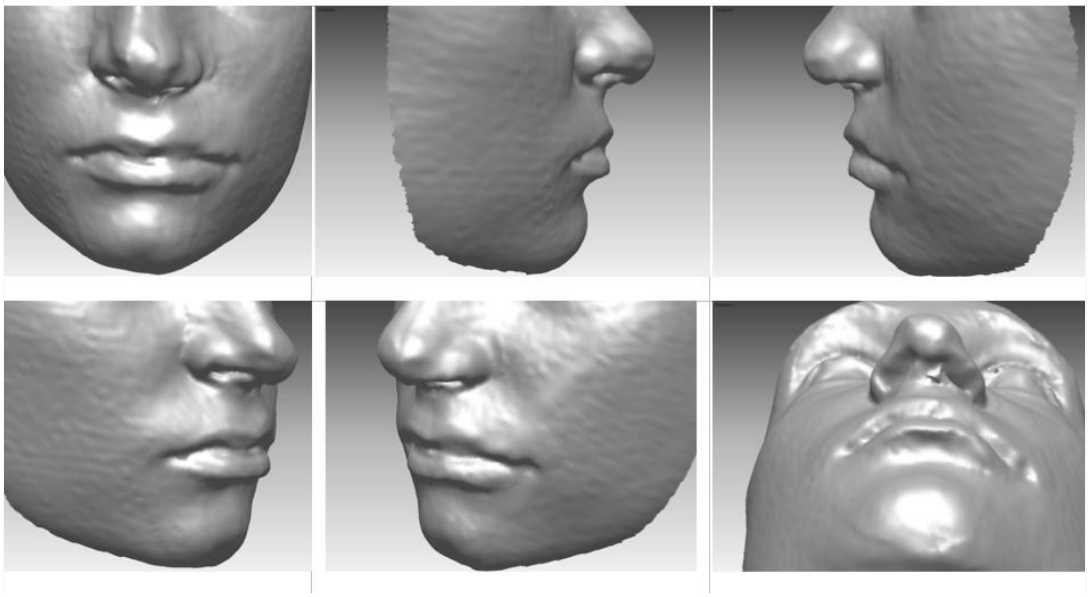


Figure 4.2 An example of the six standardised views required to assess lip phenotypes

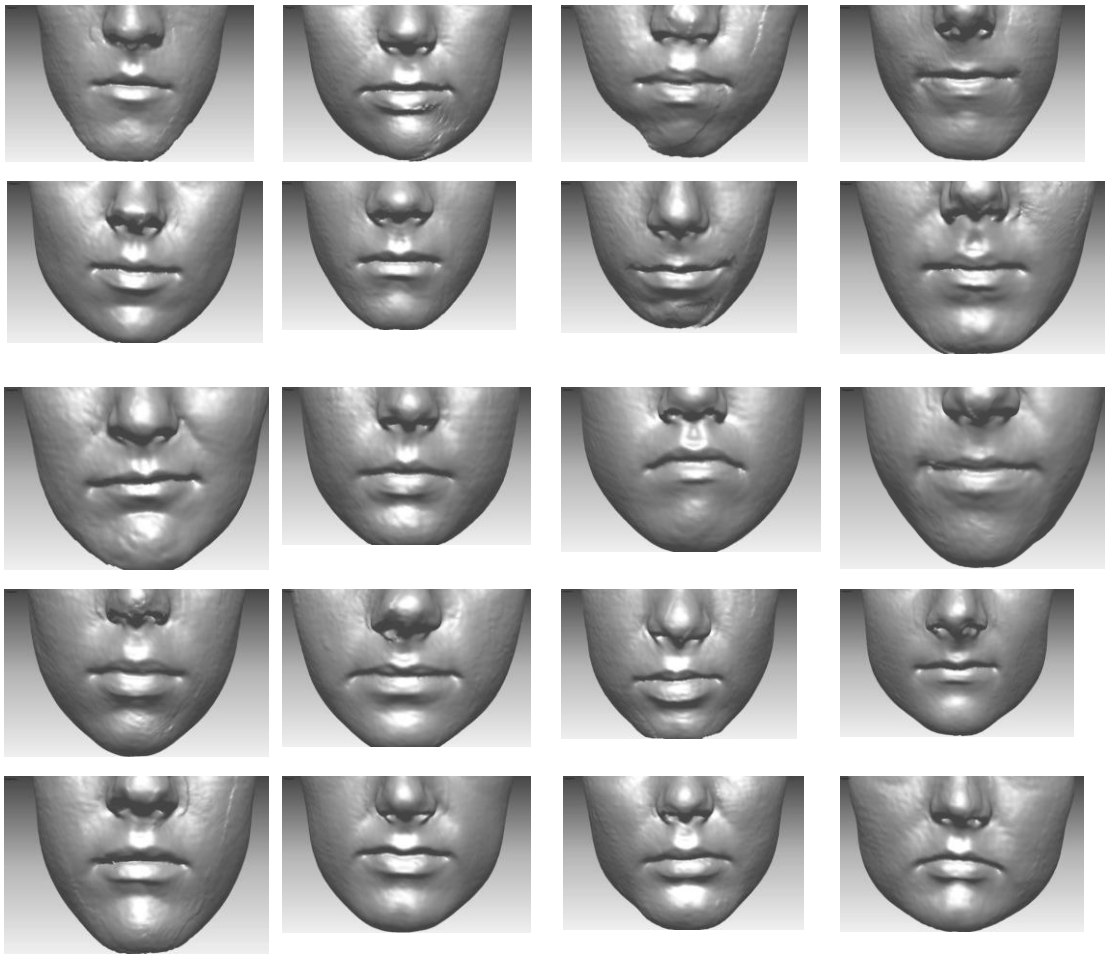


Figure 4.3 Twenty randomly selected cases were used initially to develop a rudimentary scale

The sample was increased to a hundred random images, in order to increase the number of possible variations within the population sample (2% of the population).

An assessment of skeletal pattern was also included in the scale, to rule out the possibility that some of the traits may arise as a result of variations in the position of maxillary bases. As all the images had been aligned to natural head position, an imaginary vertical line was drawn from nasion to zero meridian, and an assessment of the position of the upper lip and lower lip to this line was used to determine whether the individual was skeletal I (average), II (mandible reduced) or III (mandible prognathic).

In order to improve inter-examiner reliability, vermilion fullness could be judged with the addition of an imaginary line from the tip of the nose to soft tissues pogonion (Rickett's E-line) (Ricketts, R.M., 1968). A judgement could then be made on the relative protrusion of the lips relative to this line, with thin lips sitting behind the line, and thick lips sitting in front of the line. The draw back to this technique is that a prominent nasal tip or pogonion are likely to affect the relative position of the lips.

The scale was further refined, until a comprehensive reliable scale was produced (Figure 4.4 and Table 4.2). Intra and inter examiner reproducibility scores are presented in the results section (Table 4.3).

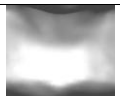
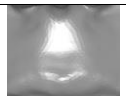
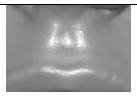
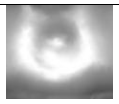
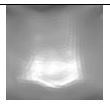
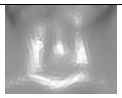
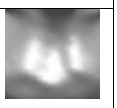
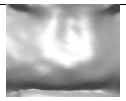
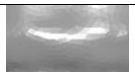
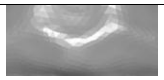
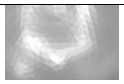
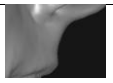
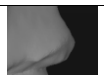
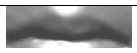
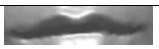
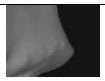
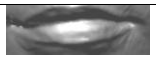
	0	1	2	3	4	5	6
Philtrum							
Philtrum Shape							
Philtrum Width							
Cupid's Bow Shape							
Nasolabial angle (Profile)							
Upper Vermilion							
Vermilion Fullness (Profile)							
Vermilion Contour							
Vermilion border							
Double Border							
Vermilion Brim (Profile)							
Vermilion Midline Groove/ Drop							
Lower Vermilion							
Vermilion Fullness (Profile)							
Vermilion Contour							
Vermilion border							
Double Vermilion Border							
Vermilion brim (Profile)							
Vermilion Groove/ Bump							
Commissures							
Commissures							
Sub-Lip							
Lip-Chin Shape (Profile)							
Mentolabial fold							
Chin dimple							
Lower lip tone (3/4 View)							
Lower lip Tone (Looking up)							
Skeletal pattern							

Figure 4.4 Classification scale for the characterisation of lip phenotypes

Table 4.2 Description of morphological traits		
Phenotype	Definition	Score
Philtrum shape	Progressive scoring of the surface of the philtrum in terms of the smoothness of the surface and the position of the largest indention from the columella to the vermilion border	0 - Smooth Philtrum
		1 - Normal gradient
		2 - Indentation near columella
		3 - Indentation in the middle
		4 - Indentation near the vermilion border
		5 - Deep groove from columella to the vermilion border
		6 - Deep groove extending through the vermilion border
Philtrum width	Three categories based on the width of the philtrum based anywhere from the columella to the vermilion border	0 - Narrow
		1 - Average
		2 - Wide
Cupid's bow shape	Progressive scoring of the Cupid's bow the higher the score the more angulated the Cupid's bow	0 - Flat
		1 - U-Shaped
		2 - Sharp V
Nasolabial angle	Columella angle which can be 90 degrees, acute or obtuse	0 – Acute
		1 – Normal
		2 - Obtuse
Upper/lower lip vermilion fullness	Progressive scoring of the fullness of the lip vermilion, not extending beyond the vermilion border (Viewed in profile)	0 - Thin
		1 - Medium
		2 - Thick
Upper lip vermilion contour	The shape of the vermilion border from the Cupid's bow peaks to the commissures	0 – Concave
		1 – Straight
		2 - Convex
		3 - Pseudo-convex
Lower lip vermilion contour	General curvature of the lower lip	0 – Narrow in midline
		1 – Straight
		2 – Curved
		3 – Markedly curved
Upper/lower lip vermilion border	Identifiable vermilion lip border with variable coverage	0 - None
		1 - Middle
		2 – Full border
Upper/lower lip double vermilion border	A ribbon of soft tissue matching the vermilion border usually lying 2mm above the border	0 - None
		1 – Present
Upper/lower vermilion brim	A small semi-circular projection at the vermilion border	0 - None
		1 - Present
Upper/lower lip vermilion midline groove/drop	The presence of different forms of midline variation Groove: grooved area (tissue deficiency) Drop: bumped area (tissue excess)	0 – None present
		1 – Notch in midline
		2 - Bunched mass in midline
Commissures	Position of the commissures in relation to the general lip line	0 - Upturned
		1 – Straight
		2 - Downturned
Lower lip-chin shape	The curvature of the sub-lip area, from the lower lip vermilion border to the chin	0 - Flat
		1 – Curved concavity
		2 – Angular concavity
		3 – Marked angular concavity
		4 – Marked angular concavity, with a convex area
Mentolabial fold	Presence of an obvious mentolabial fold	0 – Not present
		1 - Present
Chin dimple	Presence of an obvious chin dimple or cleft	0 – Not present
		1 – Dimple/cleft present
Lower lip tone / Tone-up	The assessment of the mentolabial muscle tone 1 – viewed from the ¾ angle 2 – viewed from the submentovertex (up) angle	0 - None
		1 – Slight concavity
		2 – Marked lateral square borders
		3 – Wide deep concavity
		4 - Marked tonicity with bumped areas
Skeletal pattern	The clinical assessment of the relationship of the maxilla and mandible to the cranial base	0 – Skeletal II – relative mandibular retrognathia
		1 – Skeletal I – balanced facial profile
		2 – Skeletal III – relative mandibular prognathia

4.2.5 Statistical Analysis

The data used in this study was categorical and nominal, and thus, the reliability of the features were assessed using percentage agreement scores, for which the exact same score for each feature would be required from each examiner. Kappa agreement scoring was not possible with this data, as it was not normally distributed (i.e. each category was not equally chanced, as some features were much rarer than others).

Chi-squared tests were performed to test the distribution of the data between two independent features (Table 4.5). A statistically significant p-value indicated that there was a significant difference between the expected frequency and the observed frequency in one or more categories.

4.3 Results

The sample typified normal variation in 15-year-old Caucasian children. Four thousand four hundred and seventy four children (2,514 females and 2,233 males) were classified.

4.3.1 Reproducibility

Inter and intra-examiner reliability assessments were performed on 42 subjects (Table 4.3), and percentage agreements were recorded. Intra-examiner reliability was performed by myself, two weeks apart. Inter-examiner reliability was performed between three different examiners. The first examiner was involved with the development of the classification system, whereas the second and third examiners were taught how to apply the system.

Inter-examiner reliability with the first examiner showed a good level of agreement (>70%) on many features; however, lower lip fullness, contour and groove/drop, lip-chin shape and lower lip tone agreement were poor (<50%). This could be as the first examiner was involved in the development of the scale, which was adapted several times during refinement. Lower lip vermilion contour (48%) showed particularly low reliability; however, if the scale was dichotomised to 0-2 and 3-5, the reliability was significantly improved to 67% inter-examiner reliability. The second and third examiners, who were not involved in the development of the scale, had much better reliability, with all features showing a good level of agreement (>70%).

Table 4.3 Intra and Inter examiner reproducibility

	Intra		Inter (%)		
	Percentage agreement	Kappa	Examiner 1	Examiner 2	Examiner 3
Philtrum					
Philtrum Shape	90	0.88	65	85	80
Philtrum Width	69	0.47	68	95	80
Cupid's bow shape	81	0.66	73	88	84
Nasolabial angle	88	0.80	73	93	88
Upper Lip					
Vermilion fullness	79	0.67	60	80	78
Contour	86	0.76	55	85	80
Vermilion border	86	0.54	83	83	84
Double border	83	0.60	98	93	98
Vermilion brim	90	0.77	90	93	86
Vermilion groove/drop	98	0.96	79	85	83
Lower lip					
Vermilion fullness	88	0.78	40	85	80
Contour	90	0.66	48	70	75
Vermilion border	86	0.72	80	80	87
Double border	100	1.00	80	100	100
Vermilion brim	93	0.81	65	75	93
Vermilion groove/bump	74	0.57	48	85	78
Commissures	95	0.90	60	83	82
Sub-lip					
Lip-chin shape	70	0.67	45	73	76
Mentolabial fold	88	0.71	78	85	78
Lower lip tone	79	0.69	43	83	78

There was generally good intra-examiner reliability; percentage agreement was over 70% of traits, with the exception of philtrum width (68%) and lip-chin shape (70%). Cohen's kappa coefficient was also performed, as it is generally considered to be a more robust measure than simple percent agreement calculation, as kappa takes into account the possibility of the agreement occurring by chance. Kappa scores over 0.81 are deemed to have almost perfect agreement, scores between 0.80 - 0.61 are deemed to have substantial agreement, 0.60 - 0.41 have moderate agreement, 0.40 - 0.21 have fair agreement, and scores below 0.20 have slight agreement (Landis, J.R. *et al*, 1977). According to this scale, most of the traits have substantial agreement, with

the exception of philtrum width, upper lip vermilion border, upper lip double border and lower lip groove/drop which all have moderate agreement.

The two features are difficult to classify, the philtrum width can be subjective, as the relative width of the nose and mouth can alter the examiner's perception. A possible method of improving the measurement of the philtrum width, would be to landmark the philtrum ridges, and measure the distance (objective method), however, this is also problematic, as small measurements in this region are inherently unreliable (Hennekam, R.C. *et al*, 2009). Selecting a landmark to measure from can also be problematic, as philtral ridges don't always run parallel. I.e. if measurements are taken near subnasale in an individual with divergent philtral ridges, it would give the false impression that the individual had a narrow philtrum.

Lip-chin shape has five variations, of which the second (curved concavity) and third (angular concavity) are quite difficult to differentiate. If this scale is simplified to four categories, the intra-examiner reliability increases to 85%.

Upper lip vermilion border could also be dichotomised to either no border or some/full border, this would increase the kappa agreement to 0.61.

4.3.2 Prevalence of lip morphological features

4,747 of the 5,253 subjects were categorised according to the classification scale (Figure 4.4), and the prevalence of each feature is presented (Table 4.4). The prevalence of features amongst males and females were analysed using Chi^2 statistics. A statistically significant difference was seen with twenty of the twenty-five features. An "*" indicates the feature associated sex predilection.

Table 4.4 Prevalence of features and sex dimorphisms

Feature	Classification	All		Males		Females		P-value
		N	%	N	%	N	%	
Philtrum Shape	Smooth	315	7	133	6	182	7	<0.0005
	Normal gradient	674	14	260	12	414	16*	
	Indentation near nose	333	7	117	5	216	9*	
	Indentation in middle	1991	42	923	41	1068	42	
	Indentation near VB	883	19	535	24*	348	14	
	Deep indentation	291	6	140	6	151	6	
	Deep into VB	260	5	125	6	135	5	
Philtrum Width	Narrow	542	11	180	8	362	14*	<0.0005
	Average	3266	69	1482	66	1784	71*	
	Wide	939	20	571	26*	368	15	
Cupid's Bow	Flat	329	7	127	6	202	8*	<0.0005
	U - Shaped	2539	53	1292	58*	1247	50	
	V - Shaped	1879	40	814	36	1065	42*	
Nasolabial Angle	Acute	732	15	332	15	400	16	<0.0005
	Normal	3058	65	1377	62	1681	67	
	Obtuse	957	20	524	23*	433	17	
Upper lip								
Fullness	Thin	459	10	164	7	295	12*	<0.0005
	Medium	3142	66	1427	64	1715	68	
	Thick	1146	24	642	29*	504	20	
Contour	Concave	2371	50	1003	45	1368	54*	<0.0005
	Straight	1636	35	835	37	801	32	
	Convex	350	7	208	9	142	6	
	Pseudo-convex	390	8	187	8	203	8	
Border	None	511	11	259	12	252	10	0.001
	Middle only	1640	34	712	32	928	37*	
	Full border	2596	55	1262	56	1334	53	
Double Border	None	4482	94	2090	94	2392	95*	0.020
	Present	265	6	143	6	122	5	
Brim	None	3256	69	1571	70	1685	67	0.014
	Brim	1491	31	662	30	829	33*	
Drop	None	1925	41	943	42	982	39	0.143
	Midline drop	2822	59	1290	58	1532	61	
Groove	None	3598	76	1703	76	1895	75	0.476
	Groove	1149	24	530	24	619	25	
Lower lip								
Fullness	Thin	397	8	196	9	201	8	0.388
	Medium	2947	62	1365	61	1582	63	
	Thick	1403	30	672	30	731	29	
Contour	Narrow at midline	1037	22	565	25*	472	19	<0.0005
	Straight	317	7	170	8	147	6	
	Gentle Curve	2374	50	1031	46	1343	53*	
	Curved	1019	21	467	21	552	22	
Border	None	192	4	77	3	115	5	0.141
	Middle	1499	32	706	32	793	31	
	Full	3056	64	1450	65	1606	64	
Double Border	None	2659	56	1293	58	1366	54	0.013
	Present	2088	44	940	42	1148	46*	
Brim	None	2295	48	1129	51	1166	46	0.004
	Present	2452	52	1104	49	1348	54*	
Groove	None	4108	87	1880	84	2228	89	<0.0005
	Groove	639	13	353	16*	286	11	
Bump	None	3717	78	1796	80	1921	76	0.001
	Bump	1030	22	437	20	593	24*	
Commissure	Upturned	495	10	194	9	301	12*	<0.0005
	Flat	1734	37	725	32	1009	40	
	Downturned	2518	53	1314	59*	1204	48	
Sub-lip								
Lip - Chin Shape	Flat	194	4	94	4	100	4	<0.0005
	Convex	1755	37	745	33	1010	40*	
	Angular	1841	39	917	41*	924	37	
	Angular with pronounced vermilion	560	12	315	14*	245	10	
	Angular with bump	397	8	162	7	235	9	
Mentolabial Fold	None	2665	56	1222	55	1443	57	0.064
	Present	2082	44	1011	45	1071	43	
Chin Dimple	None	3684	78	1664	75	2020	80	<0.0005
	Present	1063	22	569	25*	494	20	
Tone	None	358	7	147	7	211	8	<0.0005
	Slight convex	635	13	254	11	381	15	
	Square shaped convex	1688	36	773	35	915	36	
	Deep convex	1609	34	891	40*	718	29	
	Bilateral bumping	457	10	168	7	289	12*	
Tone - Up	None	793	17	314	14	479	19*	<0.0005
	Slight with bump	620	13	277	12	343	14	
	Square shaped convex	1370	29	634	28	736	29	
	Deep convex	1546	33	853	38*	693	28	
	Bilateral bumping	418	9	155	7	263	10	
Skeletal Pattern	Skeletal I	1207	25	407	18	800	32*	<0.0005
	Skeletal II	3031	64	1621	73*	1410	56	
	Skeletal III	509	11	205	9	304	12*	

The most prevalent feature was generally the most common amongst both sexes; however, of the less common traits this was not always the case

4.3.3 Uncommon traits

Some lip phenotypes occurred infrequently (<7%). The less common philtrum shapes were smooth (0), indentation near the nose (2) and deep crossing into the vermilion border (6). A flat Cupid's bow shape (0), double upper lip vermilion border (1), and convex vermilion contour (2) were all rare features of the upper lip. Atypical features of the lower lip vermilion were absent raised vermilion border (0), flat lip-chin shape (0), angular with bumping lip-chin shape (4), straight lower lip vermilion contour (1) and absent lip tone (0).

4.3.4 Sex Variation

Typical Female Features **Typical Males features**

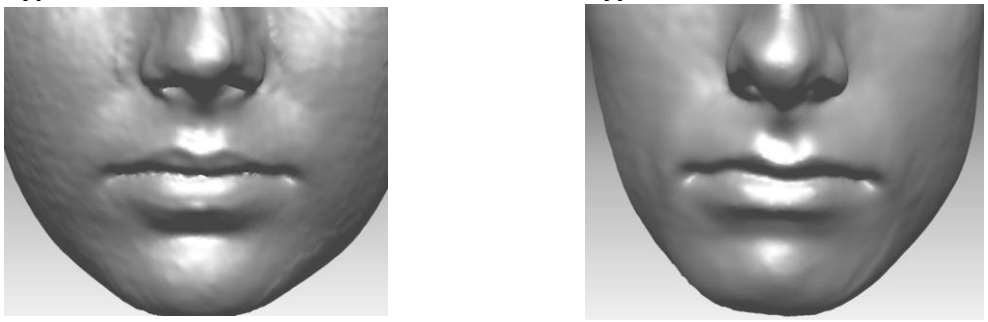


Figure 4.5 Typical female and male features

Females had a relative higher prevalence of indentation near the nose, narrow philtrum, and V-shaped or flat Cupid's bow shapes, curved lower lips with a double border and lower lip drop, whilst males had a relative higher prevalence of indentation near the vermilion border, wide philtrum and obtuse nasolabial angles, downturned commissures, a lower lip contour that narrows at the midline, a lower lip groove, a more defined lower lip tone and chin dimple (Figure 4.5).

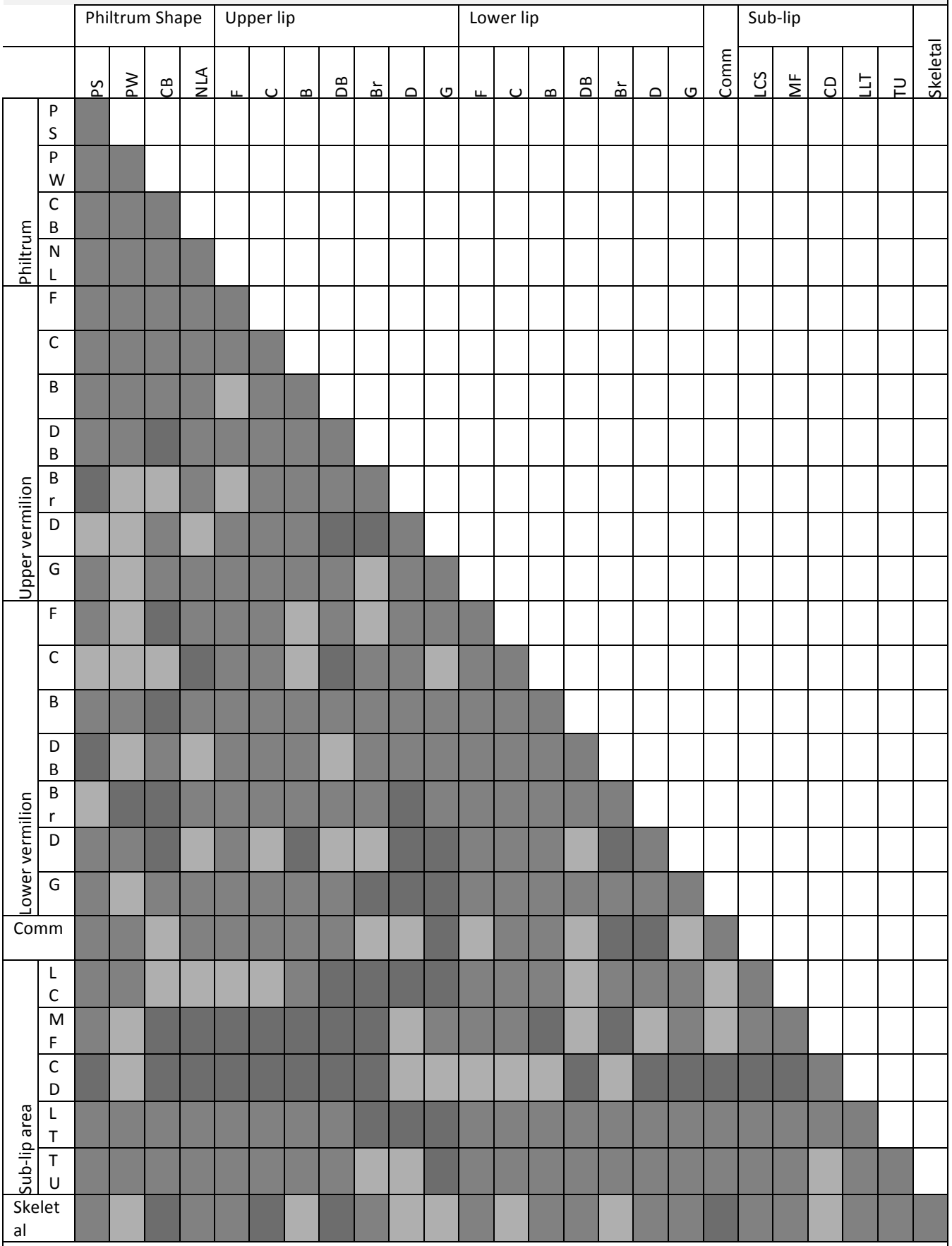
Five features did not show any sex predilection (upper lip groove/drop, lower lip border and fullness and mentolabial fold). Almost three-quarters of the male population and half of the female population had skeletal II patterns. A higher prevalence of skeletal I and III patterns were observed amongst females compared with males.

4.3.5 Morphological association

Most lip phenotypes appeared associated with other traits (Table 4.5); in particular, features of the philtrum and upper lip, which seemed to be closely associated. The mentolabial fold and chin dimple, occurred independently of other lip phenotypes.

Morphological associations between sexes also varied (Table 4.6), with females having strong associations with features of the philtrum and upper lip. Associations were also observed with the lower lip contour and Cupid's bow amongst males.

Table 4.5 Morphological associations of features amongst the 4,747 ALSPAC population

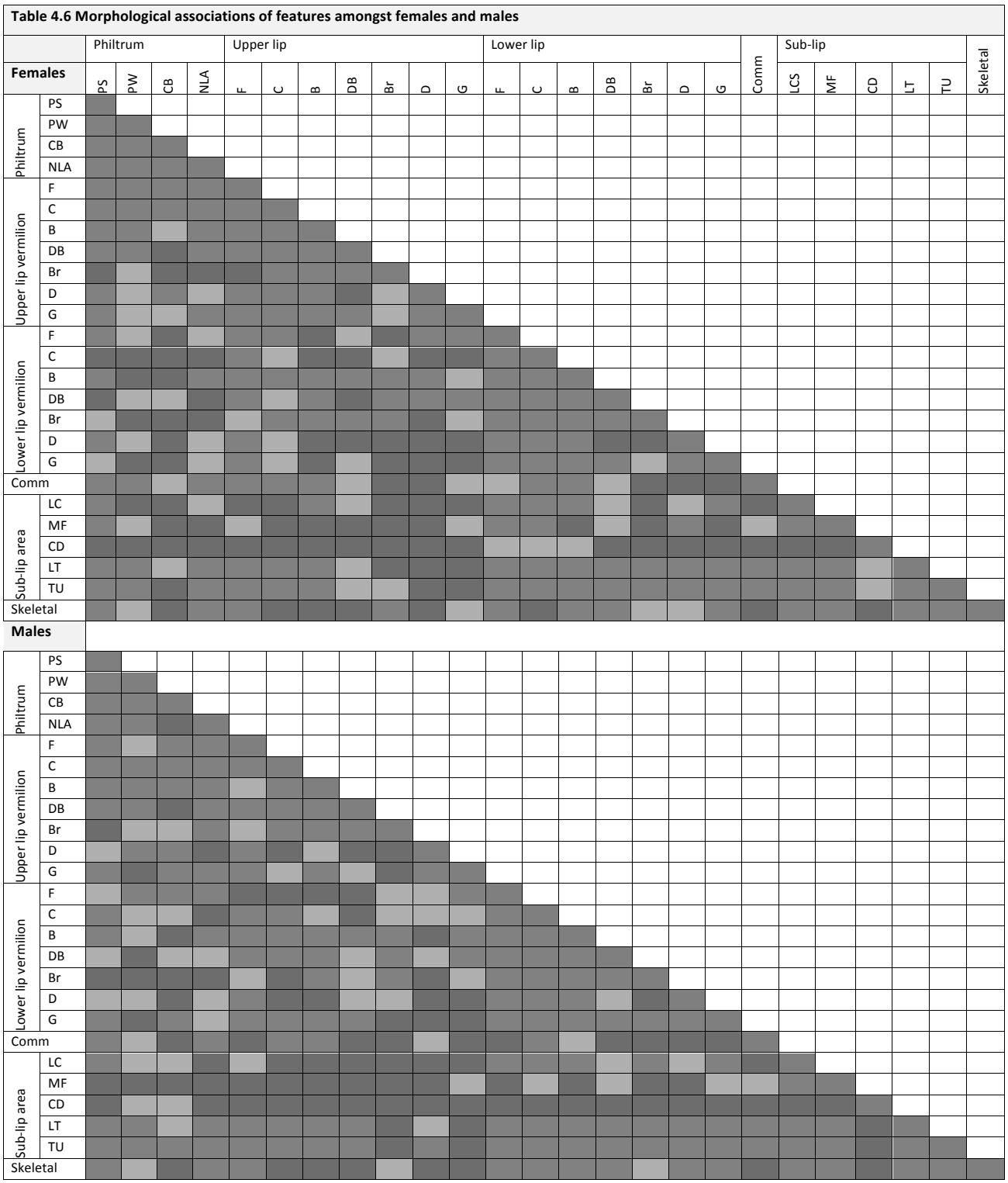


The features of the Philtrum are highly associated with other lip phenotypes (p<0.0005). The chin dimple and mentolabial fold show the least amount of association with other traits.

P-values (Green: no statistical significance; Amber: p<0.05; Red: p<0.0005).

Abbreviations: PS Philtrum shape; PW Philtrum width, CB Cupid's bow, NLA Nasolabial angle; F

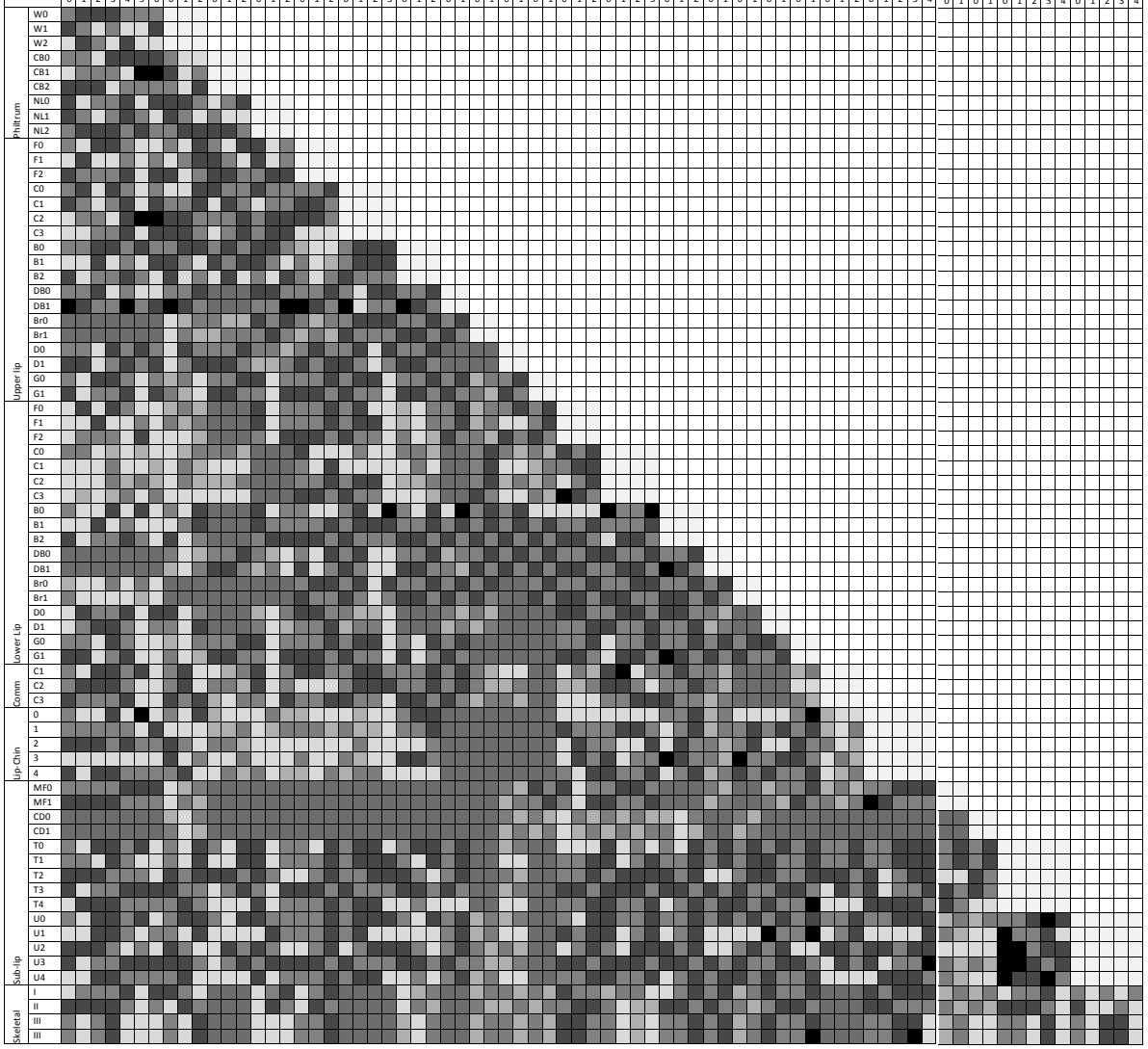
Table 4.6 Morphological associations of features amongst females and males



P-values (Green: no statistical significance; Amber: p<0.05; Red: p<0.0005).

Abbreviations: PS Philtrum shape; PW Philtrum width, CB Cupid's bow, NLA Nasolabial angle; F Fullness; C Contour; B Border; DB; Double border; D Drop; G Groove; Comm Commissures; LC Lip-chin shape; MF Mentolabial fold; CD Chin dimple; LT Lower lip tone; TU Tone-up.

Table 4.7 Morphological association of each trait



Residual scores (frequency-expected values): (Green: no statistical significance between features; Black: Reduced association p<0.0005 (<2% population); Dark blue: Reduced association p<0.0005; Light blue: Reduced association p<0.05; Grey: No significant (<10 difference between expected and actual) difference; Amber: increased association p<0.05; Red: increased association p<0.0005).

Abbreviations: PS Philtrum shape; PW Philtrum width, CB Cupid's bow, NLA Nasolabial angle; F Fullness; C Contour; B Border; DB; Double border; D Drop; G Groove; Commis Commissures; LC Lip-chin shape; MF Mentolabial fold; CD Chin dimple; LT Lower lip tone; TU Tone-up.

4.3.6 Direction of association of traits

Residual scores of lip phenotypes indicated that there were many common morphological associations (Table 4.7). For example, less distinct features, such as smooth philtrum, flat Cupid's bow shape, absent vermilion border/double borders; flat tone and lip-chin shape, absent mentolabial fold were all found to be associated with each other (Figure 4.6). These traits were also associated with a skeletal III pattern.

Figure 4.6 Features associated with smooth (left) and deep philtrum shapes (right)

More defined features, such as deep philtrum, tended to occur more frequently with increased angular features, such as a V-shaped Cupid's bow, full upper and lower lip border and upper lip double border, mentolabial fold and angular lower lip tone and lip-chin shape (Figure 4.6).

Figure 4.7 Associations with features of the inferior border of the upper lip. Upper lip groove (left), and drop (right)

Thick upper lips were more associated with straight or convex upper lip contours, an upper lip brim, upper lip border and double border. They were also associated with an upper lip groove (Figure 4.7). Average fullness of the lips on the other hand was associated with an upper lip drop. Both features of the upper lip inferior border were found to correlate with a V-shaped Cupid's bow.

Upper lip phenotypes were also found to be associated with features of the lower lip and sub-lip; thick upper lips occurred with deep concavity lower lip tone and skeletal II pattern, whilst thin upper lips were more associated with a smooth lower lip tone and skeletal III patterns. Thick upper lips were associated with thick lower lips. Thin lower lips were associated with a straight lower lip contour, a lower lip drop a mentolabial fold and a skeletal II pattern.

A narrow lower lip contour (Figure 4.8) was not found to be associated with any philtral or upper lip phenotype, but was associated with a lower lip border at the midline only, a double border, downturned commissures and defined lower lip tone.

Figure 4.8 Features associated with narrow lower lip contour and a lower lip drop Narrow lower lip contour (left) Lower lip drop (right)

There was association between lip-chin shape and lower lip tone; skeletal II patterns were associated with relative concavity of the lower lip, whilst skeletal III was associated with a flat tone/mentolabial fold. Chin dimple was associated with angular and markedly angular tone, and less associated with flat or concave lower lip tone.

4.4 Discussion

Considerable variation in normal lip morphology has been previously reported and described (Tables 1.3 and 1.4); this study has identified a variety of new morphological features, which have not been previously acknowledged (Figure 4.4 and Table 4.2). These traits are not amenable to classification by landmarking or measurement, but by anatomical shapes and surface topography using a visual/numerical scale. The scale shows a high level of reliability with most of the features in terms of intra and inter-examiner error. Inter-examiner reliability between the second and third examiners was much better, as this scale was taught to them, and therefore there appeared to be less contention whilst classifying the features. Simplifying the scale, by reducing the number of categories, could increase reliability and reproducibility of the scale.

The study presents the prevalence of lip phenotypes amongst 4,747 15-year-old individuals. There have been many previous epidemiological studies that have presented average measurements of facial phenotypes (Table 1.3). There have been fewer studies that have categorised characteristic features of the lips, those that have been undertaken, have been done on small population groups (Table 1.4).

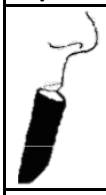
Comparisons can be drawn with regards to five of the characteristic features, which are comparable to phenotypes that have been previously described, although the methods employed by this study are not completely homologous with the other studies (Table 4.8).

4.4.1 Philtrum Shape

The variation of the philtrum shape is greater than previously reported (Mori, A. *et al*, 2005). It is evident from this study and from previous studies, that a smooth philtrum shape is a rare phenotype within the population. It had a prevalence of 7% with no apparent sex dimorphism within this population, previous studies have suggested that prevalence varies from 4.8% - 13% (Astley, S.J. *et al*, 1996, Mori, A. *et al*, 2005).

The philtrum shape identified in this classification scale (Figure 4.4) as “indentation near the vermilion border” has a similar appearance to a previously described “convex philtrum” shape (Mori, A. *et al*, 2005), both show an increased prevalence amongst males.

Table 4.8 Comparison of prevalence of lip phenotypes within this ALSPAC study and those found in previous studies

Feature	Definition	Sample	Prevalence	Reference
Philtrum shape				
	Smooth	4,747	7%, M=F	ALSPAC
		84	4.8%	(Astley, S.J. *et al*, 1996)
		109	13% M=F	(Mori, A. *et al*, 2005)
	Parallel /Normal	4,747	14%(F>M)	ALSPAC
		109	42% (F>M)	(Mori, A. *et al*, 2005)
	Convex / Indentation near VB	4,747	19% (M>F)	ALSPAC
		109	12% (M>F)	(Mori, A. *et al*, 2005)
Upper lip				
Thin		4,747	10%; (F>M)	ALSPAC
		84	6% (Likert 5)	(Astley, S.J. *et al*, 1996, Carey, J.C. *et al*, 2009)
Thick		4,747	24%; (M>F)	ALSPAC
		84	29% (Likert 1&2)	(Astley, S.J. *et al*, 1996, Carey, J.C. *et al*, 2009)
Lip-chin shape				
	Flattened	4,474	4% (M=F)	ALSPAC
		89	0	(Farkas, L.G. *et al*, 1984)
	Deep and curved / Convex	4,474	37% (F>M) 33% (M) 40% (F)	ALSPAC
		89	45% (F>M) 28% (M) 67% (F)	(Farkas, L.G. *et al*, 1984)
	Deep and indented / Angular	4,474	39% (M>F) 41% (M) 37% (F)	ALSPAC
		89	36% (M>F) 42% (M) 28% (F)	(Farkas, L.G. *et al*, 1984)
Chin dimple				
No dimple		4,747	78%; (F>M)	ALSPAC
		Germany Italy Lithuania	82% 80% 71%	(Ritz-Timme, S. *et al*, 2011)
Dimple		4,747	22% (M>F)	ALSPAC
		Germany Italy Lithuania	18% 20% 29%	(Ritz-Timme, S. *et al*, 2011)
Skeletal pattern				
Skeletal I		Caucasian	25%	ALSPAC
		European	40%	(Foster, T.D. *et al*, 1974)
Skeletal II		Caucasian	64%	ALSPAC
		European	50%	(Foster, T.D. *et al*, 1974)

4.4.2 Upper lip fullness

Lip fullness has previously been recorded using objective (vermilion height or thickness – Table 1.3) and subjective measurements (Table 1.4). This study demonstrated that thin and thick lips occur in 10% and 24% of the population, respectively. This is comparable to previous research (Table 4.8)(Astley, S.J. *et al*, 1996). The study also supports the claim that males have thicker upper lips in comparison to females (Farkas, L.G. *et al*, 1984, Mamandras, A.H., 1988, Arnett, G.W. *et al*, 1999, Kamak, H. *et al*, 2012), and that upper lips tend to be thinner than lower lips (Farkas, L.G. *et al*, 1984).

4.4.3 Lip-chin shape

Lip-chin shape has previously been described as variations in chin contour (Farkas, L.G. *et al*, 1984), an additional two were presented in this study (Figure 4.4). Comparisons can be drawn with three of the phenotypes previously described. In the previous study of 89 individuals, the flattened chin contour was described, but not observed at all, however, in this sample of 4,747 it was observed in 4% of individuals. It may not have been observed previously as it is a rare trait, and the sample size was too small. Deep and curved and deep and angulated lip chin shapes are comparable to the previous study, with the same sex distribution, with females having increased prevalence of deep and curved, and males having an increased prevalence of angulated convexity (Table 4.8).

4.4.4 Chin dimple

A population study of the prevalence of a variety of facial morphological features demonstrated that there was a difference in the prevalence of chin dimple amongst different European populations. Chin dimple was present in 22% of the ALSPAC population, this is comparable to German (18%) and Italian (20%) populations.

4.4.5 Skeletal pattern

The majority of epidemiological studies assessing the prevalence of skeletal patterns are based on measurements taken from lateral cephalograms, in this study, skeletal pattern was categorised according to the relative position of the upper lip to the chin, whilst the head is orientated in natural head position. Previous research has demonstrated that the prevalence of skeletal III pattern varies depending on ethnic

background; it has been reported to be as high as 12% amongst Chinese populations (Lew, K.K. *et al*, 1993) but less prevalent amongst European populations at 5% (Foster, T.D. *et al*, 1974). As skeletal III pattern is relatively uncommon amongst the population as a whole, the majority of previous studies have pooled males and females together, as such; there is a scarcity of research highlighting sex differences. However, one cephalometric study (comparing ANB values) suggested that sex dimorphism does exist during adolescence, with females exhibiting skeletal III patterns prior to males, due to delayed pubertal growth spurts in males (Baccetti, T. et al, 2005). Studies have also demonstrated soft tissue thickness variations amongst males and females at different ages, with females tending to have increased soft tissue thickness around the chin region at age 15years (Wilkinson, C.M., 2002, Utsuno, H. *et al*, 2010a).

A growth study of 1,594 North American Caucasians also demonstrated that the mandible reached almost its full mature size in females at 13yrs and 15 years in males (Farkas, L.G. *et al*, 1992).

Skeletal II pattern is the most common pattern amongst the ALSPAC population, and this was also observed previously in a British population (Foster, T.D. *et al*, 1974).

4.4.6 Morphological associations

This study has demonstrated that most lip phenotypes were associated with other phenotypes of the lip region (Tables 4.5 & 4.7). It has identified many more associations than the previously suggested associations, which were based on expert opinion (Table 1.5).

Many lip phenotype variations were also observed with varying skeletal patterns (Table 4.7). Individuals with a skeletal III were more associated with thin upper lips ($P<0.0005$) and thick lower lips ($P<0.0005$), and the opposite was true with skeletal II patterns. A significant difference in soft tissue thickness of the lips has been previously observed in individuals with different skeletal patterns (Kamak, H. *et al*, 2012), however, upper lip thickness was increased in skeletal III patterns compared to skeletal I or II (Kamak, H. *et al*, 2012). This variation could arise due to different methodologies employed in measuring lip thickness, that study used lateral cephalograms to measure the soft tissue thickness. One would expect to observe an increased upper lip thickness in skeletal III due to the relative retrusion of the maxillary base and maxillary incisor proclination giving rise to increased lip tissue density. Other studies have observed no

variations in lip thickness amongst different skeletal patterns (Utsuno, H. *et al*, 2010b, Pithon, M.M. *et al*, 2014).

In addition, many of these associations exist between features that may not be expected, for example V-shaped Cupid's bow and lower lip double border.

The only lip phenotypes that appear independent of most of the other lip phenotypes are chin dimple and mentolabial fold. A mentolabial fold was only observed in association with a concave lip-chin shape, angular tone and skeletal II patterns. A deep mentolabial fold has previously been observed in 40% of skeletal II patterns (Rosen, H.M., 1991, Dohvoma, C. *et al*, 1995).

4.5 Conclusions

The objectives were to:

- Develop a robust, reproducible classification system of lip phenotypes
- Measure the prevalence of lip phenotypes within a population sample of 4,747
- Assess for associations of lip phenotypes with other lip phenotypes
- Assess if there were any sex variations of lip phenotypes

A robust, reliable lip categorisation scale has been developed, which has good intra and inter-examiner reliability. It is possible to classify variation in shapes and texture of the lip region.

The prevalence of lip phenotypes has been recorded, and this demonstrates that there is considerable lip variation in a population of 15 year olds. Some rare phenotypes have been reported that have not been described previously.

Most of the lip phenotypes appear to be associated with other lip phenotypes (Table 4.5), with the exception of mentolabial fold and chin dimple. Morphological associations between sexes also vary (Table 4.6), with females having strong associations with features of the philtrum and upper lip. Males have more association with lower lip contour and Cupid's bow compared with females.

The study also demonstrated that there is sex dimorphism. Males generally have angular lower lip phenotypes and wide philtrum, whilst females have narrow philtrum and defined Cupid's bow shapes. Five features were independent of sex: upper lip groove/drop, lower lip border, fullness, and mentolabial fold.

The robustness of the lip scale and significant prevalence of lip phenotypes provides a good foundation to explore phenotype/genotype associations in populations with 3D laser scanned images.

Chapter 5: Using the lip scale for different acquisition systems

5.1 Introduction

Whilst the robustness of the lip scale provides a good foundation to explore phenotype/genotype associations in populations with 3D laser scanned images, many of the population studies use different acquisition systems, in particular 3dMD stereophotogrammetry. The technical aspects of both acquisition methods have been described previously (Chapter 1, Figure 1.8). In addition, GWAS methodologies require large population samples to ensure adequate statistical power to achieve phenotype/genotype associations.

Essentially, the two most useful modes for facial observations are those representing facial surface, with and without texture. Images viewed with the ALSPAC dataset incorporated the 'shaded' mode (grey) to derive the classification scale (Figure 4.4), as it was found that the colour texture could influence the shape of the lips. However, colour texture is often used for clinical purposes for the placement of anthropometric facial landmarks (Farkas, L.G., 1994).

Therefore, in order to draw comparisons of lip variations amongst different population groups, and to ensure a large pool of phenotypic data, it was found to be necessary to compare the similarities and differences between acquisition techniques.

5.1.1 Background

The surface detail (resolution) and texture acquired from 3D systems can vary giving rise to dissimilar images of the same subject (Ghoddousi, H. *et al*, 2007). The majority of 3D studies have involved landmarking methods, which draw upon soft tissue points of bony landmarks. There may appear to be variation in determining facial landmarks using laser scanners and stereophotogrammetry (Ghoddousi, H. *et al*, 2007, Kook, M.S. *et al*, 2014).

No previous studies appear to have assessed the surface texture of the lip region to assess subtle soft tissue variation.

5.1.2 Objective

The objective was to:

- Assess the reproducibility of the classification system with other acquisition methods

5.2 Methodology

This exploratory study was performed in the Department of Orthodontics at the University Dental Hospital in Cardiff, UK. Written invitations were sent through emails to all dental students (undergraduate and postgraduate), clinical staff (nurses and dentists) and research staff members of the Department.

5.2.1 Ethical approval

Participants were informed of the study objectives, data protection protocol, and signed consent forms. The Ethical Committee of the Dental School approved the study.

5.2.2 The sample

The intention was to include as many individuals as possible in order to provide a population with a wide range of normal lip phenotypes. The study protocol did not assume any strict inclusion criteria regarding age, ethnicity, facial morphology, or health status. However, the following exclusion criteria were set: obvious lip dysmorphology due to trauma or congenital anomaly (e.g. cleft lip), scars, and dermatological conditions affecting larger area of the lips.

A total number of 102 individuals (40 males and 62 females), aged 19-54 years agreed to take part in the study (Table 5.1).

Table 5.1 Characteristics of the study sample

		Students	**Staff**	**Total**
Gender	Males	35	5	40
	Females	36	26	62
Age (years)	Mean ± SD	24.2 ± 4.3	35.0 ± 9.8	27.5 ± 8.1
Ethnicity	Caucasian	46	28	74
	Non-Caucasian	25	3	28

5.2.3 Data capture

3D facial images were obtained using well-published acquisition techniques (Kau, C.H. *et al*, 2005b, Kau, C.H. *et al*, 2008). Stereophotogrammetry was performed using 3dMD face dynamic system (3dMD Inc., Atlanta, GA, USA). After acquiring the facial image,

which took approximately 1.5ms, the image was viewed in the proprietary software; to check the quality against previously described criteria.

Facial images were imported into commercial software Rapidform® 2006 software (INUS Technology Inc. Seoul, South Korea). Laser scans were processed in three steps using in-house developed subroutine for Rapidform 2006 (Kau, C.H. *et al*, 2010).

5.2.4 Phenotyping

A total of 408 3D facial images were created to assess the lip vermilion morphological traits. Each participant had a 3D laser and 3dMD stereophotogrammetry image created, and for each capture method, an image with (colour) and without colour texture (grey) was generated (Figure 5.1). Therefore, each individual had four images produced

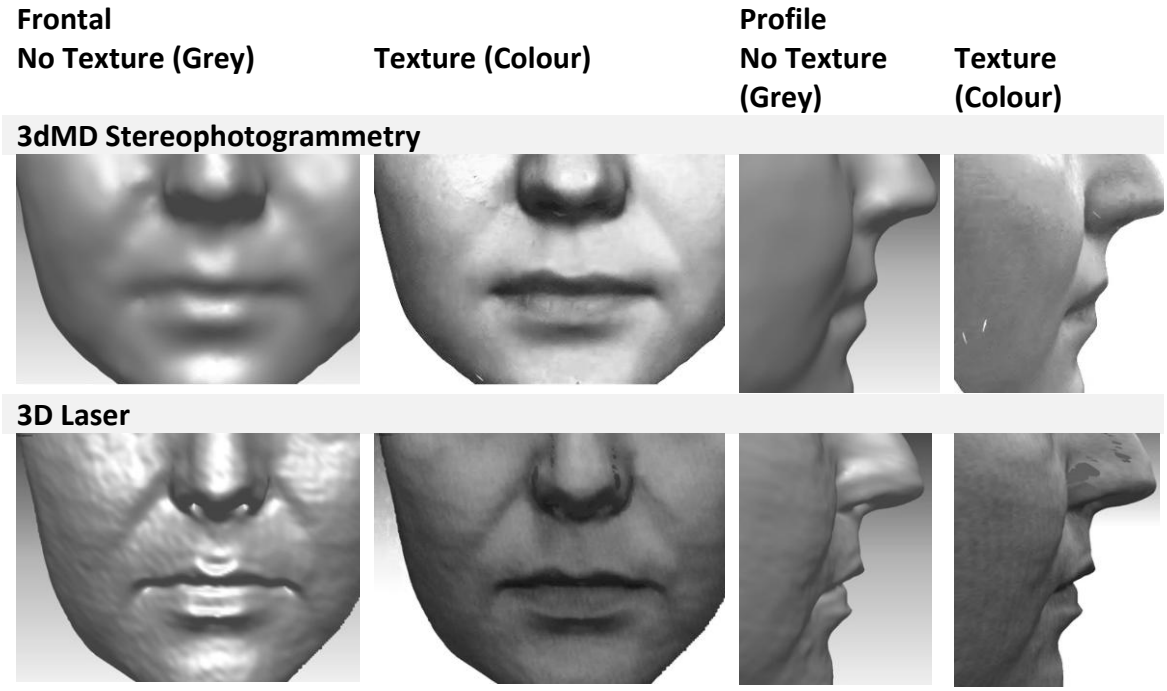


Figure 5.1 Comparison of images produced from 3D laser and 3dMD
Images with texture (colour) and without texture (greyscale).
The top row is produced using 3dMD stereophotogrammetry, the bottom row is produced using 3D laser scanning. (Viewed in Rapidform 2006).

5.2.5 Classification of Morphological Features

The lip phenotype classification system deployed has been previously described in detail (Chapter 4). Lip phenotypes were recorded for each participant, using each of the acquired facial images (Figure 5.1).

5.2.6 Statistical Analyses

Percentage agreement scores and Kappa statistics were calculated to determine reproducibility and reliability of the acquisition methods. Laser images without texture (Laser grey) was assumed to be the gold standard, and reproducibility of the other methods were tested against it.

5.3 Results

All the facial scans were analysed by one examiner (CWN), and phenotyping was performed according to the classification scale (Figure 4.4 & Table 4.2). All 102 scans from each acquisition were analysed independently of the other acquisition methods, in order to ensure blinding and eliminate any chance of bias.

5.3.1. Intra-examiner reliability of the gold standard capture method

Intra-examiner reliability was performed on a randomly selected group of 40 of the laser grey images at a two-week interval. This was to ensure reproducibility and reliability of the gold standard. The scores (Table 5.2) are comparable to those performed with the ALSPAC data (Table 4.4).

5.3.2. Reproducibility and reliability of the classification scale between laser grey and the other acquisition methods

Percentage agreement and kappa scores between the laser grey (gold standard) and the other acquisition methods were calculated and recorded (Table 5.2).

Generally agreement scores between laser grey and the three other methods were poor. Laser with colour texture had the best agreement with the gold standard, however, most of the scores were below 70%, with the exception of nasolabial angle, upper lip double border, lower lip border, lower lip double border, groove/drop and brim, and mentolabial fold and skeletal pattern. However, the kappa scores were all relatively poor. Philtrum shape was particularly poor (28% and 0.12 kappa)

3dMD with texture (colour) was superior to 3dMD without texture (grey) and had better scores than the laser colour for philtrum shape and Cupid's bow shape, although these were far inferior to the laser grey agreement scores.

It was not possible to detect some of the lip phenotypes with the 3dMD camera, these include the double vermilion border, groove/drop, brim and tone. The kappa scores for all of these traits are therefore particularly low.

Table 5.2 Reliability and reproducibility of various acquisition methods compared with the gold standard (laser grey)

Lip phenotypes		Capture Method						
		Laser			3dMD			
		Without texture (Grey) (Intra-examiner)	With texture (Colour)		Without texture (Grey)		With texture (Colour)	
		%	%	K	%	K	%	K
Philtrum	Shape	75	28	0.12	40	0.28	60	0.51
	Width	75	60	0.15	56	0.02	53	0.20
	Cupid's Bow	80	59	0.23	45	0.03	62	0.28
	Nasolabial Angle	75	73	0.42	61	0.20	59	0.15
Upper Lip	Fullness	65	56	0.22	49	0.12	54	0.15
	Contour	75	47	0.22	43	0.01	26	0.05
	Border	70	45	0.14	21	0.03	35	0.03
	Double	90	74	0.17	75	0.01	67	0.10
	Groove/Drop	80	47	0.22	51	0.03	52	0.19
	Brim	70	66	0.28	58	0.00	58	0.00
Lower Lip	Fullness	75	70	0.32	51	0.04	55	0.04
	Border	75	75	0.20	16	0.01	43	0.08
	Contour	85	67	0.38	44	0.11	49	0.14
	Double	95	75	0.20	25	0.01	34	0.05
	Groove/Drop	90	75	0.24	73	0.04	70	0.01
	Brim	85	85	0.19	10	0.00	10	0.00
Commissure		70	68	0.48	43	0.08	47	0.14
Sub-lip	Chin shape	65	60	0.40	49	0.20	42	0.19
	Mentolabial	80	72	0.41	62	0.22	66	0.31
	Tone	65	57	0.41	30	0.11	31	0.11
	Tone (up)	70	34	0.16	22	0.06	26	0.08
Skeletal		70	74	0.47	71	0.41	63	0.29

5.4 Discussion

This study has demonstrated that there is significant variation in the interpretation of the images produced by the four different capture methods. Previous studies have also confirmed that there is significant variation in determining facial landmarks captured using stereophotogrammetry and laser scanners. However, there were no significant variations between laser scanned images and those measured using direct anthropometry or CT scanners (Ghoddousi, H. *et al*, 2007, Kook, M.S. *et al*, 2014).

It is possible to compare the characteristics of the images produced by the four capture methods (Figure 5.1). Images produced with 3dMD stereophotogrammetry (top row) have a much softer, rounded appearance, (this is particularly evident when comparing the profile views) with the 3D laser images (bottom row), which have a more angular appearance. It is possible to see that the 3D laser image is able to capture the appearance of a brim on both the upper and lower lips, this is not captured at all in the 3dMD image.

In the 3D laser image without texture, it is possible to see a roped appearance to the lower lip vermilion (lower lip double vermilion border), it is somewhat visible in the laser colour image, but not visible at all in the 3dMD image. In fact, it is not possible to accurately locate the lip vermilion borders at all in the 3dMD image without texture.

This can be further demonstrated with a superimposition of two scans of one individual taken with the 3dMD and 3D laser cameras (Figure 5.2).

There is generally good agreement of the two surfaces of the face in all areas, except the lip vermilion border and brim, which could not be captured by the 3dMD system (Figure 5.2).

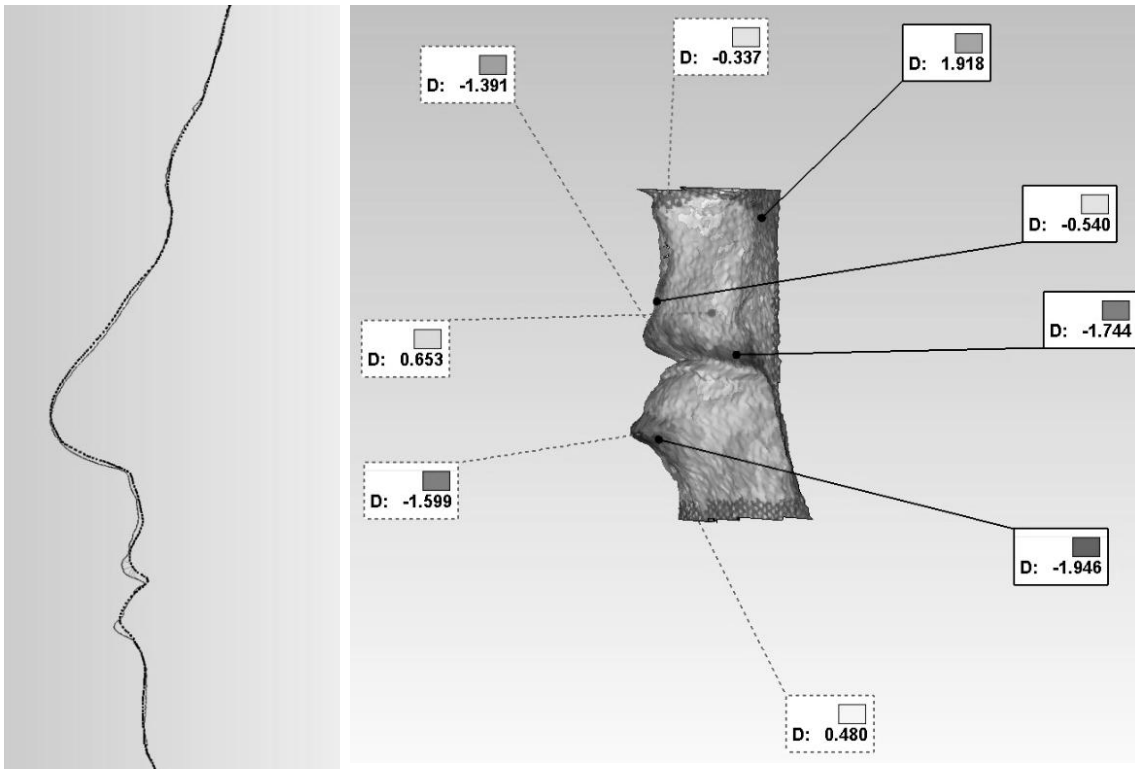


Figure 5.2 Comparison of surface detail between laser scanning and 3dMD
The line shows good reproducibility for all areas of the face, except the lips; with the 3dMD image unable to capture the finer details of the vermilion border and brim.

There is a deficit of between 1.6 - 1.9mm around the lower lip vermilion border and between 0.5 – 1.4mm around the upper lip vermilion border between the two systems. The 3D laser-scanned images are superior at capturing the finer details around the lip vermilion.

This variation is likely to arise as a result of the difference between the ways that the two cameras capture an image. Images taken with a 3dMD Stereophotogrammetry camera are captured using an arrangement of cameras, which are configured as a stereo pair to capture several images of an object. These are then amalgamated together to a best fit, and this is what gives the perception of depth to the image (Heike, C.L. *et al*, 2010). Images produced with 3D Laser scans are captured by a laser beam, which deflects from a mirror onto the scanning object. It is able to measure between 20,000 to 120,000 points on an object, and uses these to construct a 3D image. It is this technology that can capture the surface topography of the face that is necessary to capture certain features such as the vermilion borders, philtral ridges and brim (Kau, C.H. *et al*, 2005b, Toma, A.M. *et al*, 2009).

5.4.1 Adaptation of the classification scale for different capture methods

In order to interpret variations in lip phenotypes using different capture methods, it would be necessary to adapt the scale, in order for comparisons to be drawn (Table 5.3).

Table 5.3 Interpretation of using different capture methods

Philtrum Shape	It was not possible to detect the indentation (scores 2, 3, 4) with 3dMD. The scale should be simplified to 0 (smooth), 1 (normal), 5 (deep) only.
Philtrum width	Philtrum width was distorted by colour, and tended to appear either more narrow or wide and less likely to score as average.
Nasolabial Angle	Nasolabial angle appears more acute with grey images.
Cupid's Bow	Cupid's bows are less likely to appear flat in grey images, compared with colour images.
Lip fullness	Lips appeared fuller in grey images compared with colour images. This may be due to difficulty of finding where the lip starts/ends with grey images.
Lip contour	Colour images distort the contour shape of the lips, as the eye is drawn to the shape of the red vermilion, rather than the contour of the textured border.
Lip border	Grey images indicate borders/edges; whereas texture images give the illusion that a full border was present, even though there was no raised margin.
Lip brim	It is not possible to detect a brim with 3dMD images, due to the reduced surface resolution (Figure 5.2).
Upper lip double border	This is a subtle raised margin, which is present above the vermilion border. It is not picked up with the 3dMD grey images, but it is possible to detect with colour images.
Commissures	Colour images affect the impression of the commissures of the lips; colour images tended to look more upturned, whilst grey were more downturned.

5.5 Conclusion

The objective was to:

- Assess the reproducibility of the classification system with other acquisition methods

The objective of this study was to assess the reproducibility of the classification system with other acquisition methods. This study highlighted the limitations of comparing data acquired from these two different 3D capture systems, and that texture can also impact on the image produced.

Laser surface scanning constructed images with superior surface resolution, which was best viewed in greyscale format; whilst in comparison, stereophotogrammetry produced excellent skin colour images but had poorer surface resolution.

Chapter 6: Genome-Wide Association Study

6.1 Introduction

Facial development is influenced by genetic (Grosen, D. *et al*, 2010a, Peng, S. *et al*, 2013) and environmental factors (Abel, E.L., 1995, Honein, M.A. *et al*, 2007, Al Ali, A. *et al*, 2014). Developmental biological studies, based on animal models, have demonstrated that craniofacial development is tightly regulated by chemical mediators (Table 2.1). The previous chapters have demonstrated that it is possible to assess subtle features of the lip and surrounding region. It was also demonstrated in Chapter 4 that considerable individual variations exist, which may be attributable to genetic variation.

Genome-wide association studies have demonstrated successes in assigning genetic association to population variations with anatomical bony landmarks (Paternoster, L. *et al*, 2012). This chapter explores the prospect of assigning genetic association to subtle soft tissue variations of the lips and surrounding region.

Genome-wide association studies involve two parts, the first part being the discovery phase, whereby a population is assessed for genetic associations, and the second being the replication phase, where hypotheses from the first phase is tested. Ideally the replication sample should be from the same population group, in order to confirm the same effect as the target population, and also the sample should be larger than the initial study, so as to confirm any false-positive results (Bush, W.S. *et al*, 2012). With replication, it is important for the study to be well powered to identify spuriously associated SNPs where the null hypothesis is most likely true.

Once an effect is confirmed in the target population, other populations may be sampled to determine if the SNP has an ethnic-specific effect. Replication of a significant result in an additional population is sometimes referred to as generalisation, meaning that the genetic effect is of general relevance to multiple human populations.

6.1.1 Objective

The objective was to:

- Perform a genome-wide association study (GWAS) of lip phenotypes

6.2 Methodology

The sample of individuals from the ALSPAC dataset (Chapter 4) was used for the discovery phase. Imputed genotype was available for 8,365 individuals and phenotype for 4,747 indivi3duals. A total of 3,687 had both imputed genotype and phenotype available for testing (Table 6.1).

Table 6.1 Distribution of the ALSPAC sample

	Data available
Total ALSPAC sample	14535
Genotyped participants	9912
Genetic imputation	8365
Attended for facial scans	5253
Phenotyped	4747
Genetic imputation & phenotype	3687

6.2.1 Genetic data

9,912 participants were genotyped with the Illumina HumanHap550 quad genome-wide SNP genotyping platform by 23andMe subcontracting the Wellcome Trust Sanger Institute (Cambridge, UK) and the Laboratory Corporation of America (Burlington, NC, US). Individuals were excluded on the basis of having incorrect sex assignments; minimal or excessive heterozygosity (<0.32 and >0.345 for the Sanger data and <0.31 and >0.33 for the LabCorp data); disproportionate levels of individual missingness (>3%); evidence of cryptic relatedness (>10% IBD); and being of non-European ancestry. The resulting data set consisted of 8,365 individuals.

Of the 8,365 ALSPAC genotyped, phenotyped data was available for 3,687 (1,737 males and 1,950 females) individuals, who were genotyped with either the Illumina 317K or 610K genome-wide SNP genotyping platforms by the Wellcome Trust Sanger Institute (Cambridge, UK) and the Centre National de Genotypage (Evry, France). A common set of SNPs (present in both genotyping platforms) were extracted and the resulting raw genome-wide data were subjected to standard quality control methods (Paternoster, L. *et al*, 2012).

SNPs with a minor allele frequency of <1%, call rate of <95%, not in Hardy-Weinberg equilibrium and imputation quality R-squared < 0.3 were excluded.

6.2.2 Statistical analysis

Genome association was tested using regression analysis in Mach2QTL (Li, Y. *et al*, 2010). Logistic regression was performed for binary traits using mach2dat, ordinal regression was performed for the ordinal traits using r- statistics, and multinomial regression was performed using r – statistics for the multinomial trait philtrum shape (Table 6.2).

The genome wide association was performed by a PhD student (Mary Ward) in Bristol University, under the supervision of Dr Lavinia Paternoster.

Table 6.2 Statistical analysis

Binary	Upper lip border; Double border; Groove; Drop; Brim; Mentolabial fold; Chin dimple
Ordinal	Philtrum width; Cupid's bow shape; Upper lip contour; Lower lip border; Lower lip contour; NLA; Fullness; Lip-chin shape; skeletal pattern; Commissures; Tone; Tone up
Multinomial	Philtrum shape

6.3 Results

Bonferroni correction is an approach to correct for multiple testing, and adjusts the alpha value from a = 0.05 to a = (0.05/k) where k is the number of statistical tests conducted. In this GWAS there were around 500,000 common SNPs, statistical significance of a SNP association was therefore set at 0.05/500,000 = 1e-7.

This correction is the most conservative method, as it assumes that each association test of the 500,000 SNPs is completely independent of other SNPs, which is an untrue assumption to make due to linkage disequilibrium among GWAS markers (Bush, W.S. *et al*, 2012).

Genome-wide significance was reached with two gene associations, *DOCK1* with the phenotype chin dimple ($P=2.3x10^{-8}$), and gene *CDH4* with mentolabial fold ($P=9.7x10^{-8}$). Close GWAS association hits were also reached with 29 SNPs and 18 different lip phenotypes a number of other genes (Table 6.3).

Table 6.3 Results of the discovery phase genome-wide association study (GWAS)

Feature	P-Value	Reference SNP	Cytogenetic location	Gene
Nasolabial Angle	$2.8x10^{-7}$	rs7548604	1q42.2	*AGT/CAPN9*
Upper lip Fullness	$3.6x10^{-7}$	rs584267	3q26.31	*NLGN1*
	$5.7x10^{-7}$	rs687542	8q22.2	*NCALD*
	$8.7x10^{-7}$	rs4273915	9q32-q33.3	*PTGS1*
Upper Lip Contour	$8.6x10^{-7}$	rs10820934	9q22.2	*SPTLC1*
Upper lip vermilion border	$4.9x10^{-7}$	rs11771794	7p12.1	*COBL*
	$4.6x10^{-7}$	rs1317641	Xp21.2	*DMD*
	$8.5x10^{-7}$	rs12693640	2q32.3	*TMEFF2*
	$9.3x10^{-7}$	rs6791077	3q23	*CLSTN2*
Upper lip double vermilion border	$3.0x10^{-7}$	rs2365281	7q36	*RNF32*
	$5.4x10^{-7}$	rs9386046	6q24.2	*PHACTR2*
	$6.3x10^{-7}$	rs916828	11p15.1	*ABCC8*
Upper lip drop	$5.0x10^{-7}$	rs1610305	20p13	*STK35*
Upper lip groove	$8.3x10^{-7}$	rs4601553	1q42.11 1q42.13	*CDC42BPA / ZNF678*
Lower lip Fullness	$9.4x10^{-7}$	rs1035055	2q24.2	*TANC1*
Lower Lip Contour	$7.0x10^{-7}$	rs6971502	7p14.1	*CDK13*
Lower lip vermilion border	$5.3x10^{-7}$	rs10463015	5q35.1	*UBTD2*
Lower lip double vermilion border	$1.8x10^{-7}$	rs6439121	3q21.3	*EEFSEC*
	$2.4x10^{-7}$	rs2955102	3q21	*RUVBL1*
	$7.9x10^{-7}$	rs5933620	Xp11.22	*KDM5C*
Lower lip brim	$5.1x10^{-7}$	rs17035178	2p14	*CNRIP1/ PPP3R1*
Lower lip drop	$8.3x10^{-7}$	rs17629330	4p15.1	*LOC105374557*
Lower lip groove	$4.0x10^{-7}$	rs231361	11p15.5	*KCNQ1*
Mentolabial fold	$9.7x10^{-8}$	rs6061441	20q13.3	*CDH4*
	$8.0x10^{-7}$	rs4820309	22q11.22	*TOP3B*
Chin dimple	$2.3x10^{-8}$	rs11017876	10q26.13-q26.3	*DOCK1*
	$8.0x10^{-7}$	rs16889815	5p13.3	*ZFR*
Lower lip tone (UP)	$3.3x10^{-7}$	rs7011739	8q24.21	*FAM84B*
Skeletal Pattern	$5.0x10^{-7}$	rs12767587	10p12.1	*GPR158*
	$7.0x10^{-7}$	rs402800	3q26.31	*TNFSF10 / AADACL1*
	$7.0x10^{-7}$	rs17752647	2q22.1	*NXPH2*

6.4 Discussion of the discovery phase

Two associations met the stringent Bonferroni correction; chin dimple and *DOCK1* gene ($2.3x10^{-8}$) and mentolabial fold and *CDH4* gene ($9.7x10^{-8}$). A number of other close hit associations arose, which are worthy of discussion.

6.4.1 Philtrum

The only GWAS association with features of the philtrum was with nasolabial angle and SNP rs7548604 ($P=2.8x10^{-7}$), which is located within 1q42.2. The nearest gene is *AGT/CAPN9*, which encodes Angiotensinogen protein. *AGT* is an essential component of the renin-angiotensin system, and regulates blood pressure, body fluid and electrolyte homeostasis. Mutations in this gene are associated with a susceptibility to essential hypertension, and can cause renal tubular dysgenesis. Defects have also been associated with non-familial structural atrial fibrillation, and inflammatory bowel disease.

Mutations in the 1q42.2 locus have previously been associated with chondrodysplasia punctata (Warman, M.L. *et al*, 2011). Individuals with Binder syndrome have characteristic facial features, including a skeletal III pattern, flat facial profile, convex upper lip and acute nasolabial angle. Authors have previously postulated that individuals with Binder's are likely to have a mild form of chondrodysplasia punctata (Levaillant, J.M. *et al*, 2009)

1q42 is also a breakpoint hotspot with individuals either possessing duplications or deletions at the site, which result in altered facial characteristics affecting the nose (Nevado, J. *et al*, 2014).

In addition, the gene LEFTY1 lies within the locus 1q42.12. This gene is responsible for left/right patterning, and was found to be associated with face shape, in particular mid-face A-P projection (Miller, S.F. *et al*, 2014a). LEFTY1 has also been associated with dental phenotypes: centre-line asymmetry (p=0.008) and class 2 div 2 incisor relationships (p=0.049) (Weaver, C.A., 2014).

6.4.2 Upper lip

6.4.2.1 Upper lip fullness

Upper lip fullness had three associations; rs584267 ($3.6x10^{-7}$) located within 3q26.31, rs687542 ($5.7x10^{-7}$) located within 8q22.2 and rs4273915 ($8.7x10^{-7}$) located within 9q32-q33.3.

The gene residing within SNP rs687542 is *NCALD*; it encodes a family of calcium-binding proteins. It is thought to be a regulator of G protein-coupled receptor signal transduction. It is also located within the 8q22.2 region, which has been demonstrated to be a NSCLP susceptibility locus (Leslie, E.J. *et al*, 2016, Beaty, T.H. *et al*, 2016). In addition, the gene *VPS13B* is located within 8q22.2, and mutations result in Cohen syndrome, which has a short upper lip phenotype.

The gene residing within SNP rs584267 is *NLGN1*, it encodes for a cell surface protein involved in cell-cell-interactions via its interactions with neurexin family members. It plays a role in synapse function and synaptic signal transmission, and is required to maintain wakefulness quality and normal synchrony of cerebral cortex activity (El Helou, J. *et al*, 2013).

The gene residing within SNP rs4273915 is *PTGS1*, it encodes Prostaglandin G/H synthase 1, which is an enzyme that catalyses the conversion of arachidonate to prostaglandin during the COX cycle. The encoded protein regulates angiogenesis in endothelial cells, and is inhibited by non-steroidal anti-inflammatory drugs. The protein also promoted cell proliferation during tumor progression (Axelsson, H. *et al*, 2005).

6.4.2.2 Upper lip contour

One association was found between upper lip contour and rs10820934 ($8.6x10^{-7}$). The gene residing within SNP rs10820934 is *SPTLC1*, which is located within 9q22.2. *SPTLC1* encodes the SPTLC1 protein, *a* member of the class-II pyridoxal-phosphate-dependent aminotransferase family. It is the key enzyme in sphingolipid biosynthesis. Mutations in this gene were identified in patients with hereditary sensory neuropathy type 1.

It is also located within the 9q22 locus, which has been previously strongly associated as a cleft susceptibility locus (Beaty, T.H. *et al*, 2013, Ludwig, K.U. *et al*, 2014, Beaty, T.H. *et al*, 2016).

6.4.2.3 Upper lip vermilion border

Upper lip vermilion border had four near hit associations; rs11771794 ($4.9x10^{-7}$) located within 7p12.1, rs1317641 ($4.6x10^{-7}$) located within Xp21.2, rs12693640 ($8.5x10^{-7}$) located within 2q32.3 and rs6791077 ($9.3x10^{-7}$) located within 3q23.

The SNP rs12693640, which is in the cytogenic region 2q32.3, is closest to gene *TMEFF2.* It encodes the Tomoregulin-2 protein, which is a member of the tomoregulin family of transmembrane proteins. This protein functions as both an oncogene and a tumour suppressor depending on the cellular context (Chen, X. *et al*, 2011).

This SNP is also located near the cleft susceptibility gene *SATB2* (2q33.1). Although initial studies showed the CLP susceptibility locus at two translocation break breakpoints on 2q32 (Brewer, C.M. *et al*, 1999), a meta-analysis of GWAS studies of CLP indicated the whole 2q32-q35 region as a clefting susceptibility locus (Marazita, M.L. *et al*, 2004). *Satb2* plays an important role in tooth and craniofacial development (Dobreva, G. *et al*, 2006), and studies have shown that complete functional loss of *Satb2* leads to increased apoptosis in the developing jaw and subsequent down-regulation of the expression of important craniofacial genes *Pax9*, *Alx4* and *Msx1* (Britanova, O. *et al*, 2006).

The *SATB2* gene has also been associated with proclined upper incisors ($p<0.0005$) (Weaver, C.A., 2014); and this association could be significant with the association of vermilion border. Mutations in the *SATB2* region are associated with glass syndrome, which is characterised by CP, micrognathia and dysmorphic facial features (Brewer, C.M. *et al*, 1999).

The SNP rs11771794 ($4.9x10^{-7}$) located within 7p12.1 lies in closest proximity to the gene *COBL,* which encodes the cordon-bleu protein. It is a member of the WH2 repeat proteins. It has been shown to play an important role in the reorganisation of the actin cytoskeleton (Ahuja, R. *et al*, 2007). It regulates neuron morphogenesis and increases branching of axons and dendrites. It also have a role in patterning and morphogenesis, in particular neural tube formation (Carroll, E.A. *et al*, 2003). It has also been show to be a Type 1 diabetes susceptibility gene (Winkler, C. *et al*, 2012).

The SNP rs1317641 ($4.6x10^{-7}$) located within Xp21.2 lies in closest proximity to *DMD.* This gene is part of the dystrophin-glycoprotein complex (DGC) and is found at the inner surface of muscle fibres, bridging the inner cytoskeleton (F-actin) and the extra-cellular matrix. It has an essential role in the regulation of stem cell polarity and

asymmetric division (Dumont, N.A. *et al*, 2015). Mutations cause Duchenne (DMD) and Becker (BMD) muscular dystrophies, which are a recessive, fatal, X-linked disorder occurring at a frequency of about 1 in 3,500 newborn males.

The SNP rs6791077 ($9.3x10^{-7}$) located within 3q23 lies in closest proximity to the *CLSTN2* gene, which encodes the protein Calsyntenin 2, which is localised in the postsynaptic membrane of excitatory central nervous system (CNS) synapses (Hintsch, G. *et al*, 2002).

6.4.2.4 Upper lip double vermilion border

Upper lip double border had three associations; rs2365281 ($3x10^{-7}$) located within 7q36, rs9386046 ($5.4x10^{-7}$) located within 6q24.2 and rs916828 ($6.3x10^{-7}$) located within 11p15.1.

The SNP rs2365281 ($3x10^{-7}$) located within 7q36 lies in closest proximity to the gene *RNF32,* which encodes the ring finger protein. RING finger proteins are known to be involved in protein-DNA or protein-protein interactions. They play a role during spermatogenesis, most likely in spermatocytes and/or in spermatids (Van Baren, M.J. *et al*, 2002). They may also have a role to play in preaxial polydactyly (Li, H. *et al*, 2009).

The region is also in close proximity to the *SHH* gene (7q36.3) which is a known to have an important role in patterning during facial development (Jeong, J. *et al*, 2004, Stanier, P. *et al*, 2012), is a cleft susceptibility locus (Zhang, Z. *et al*, 2002), and has been demonstrated to be associated with retroclined maxillary central incisors (p=0.048) (Weaver, C.A., 2014)

The SNP rs9386046 ($5.4x10^{-7}$) located within 6q24.2 lies in closest proximity to the gene *PHACTR2,* which encodes the phosphatase and actin regulator enzyme. This enzyme is associated with nuclear scaffold in proliferating cells (Allen, P.B. *et al*, 2004) including regulatory roles in synaptic activity and dendritic morphology.

The SNP rs916828 ($6.3x10^{-7}$) located within 11p15.1 lies in closest proximity to the *ABCC8, which encodes the ATP-binding cassette transporter sub-family C member,* 8 protein. ABC proteins transport various molecules across extra and intra-cellular membranes, and functions as a modulator of ATP-sensitive potassium channels and insulin release. Mutations have been associated with non-insulin-dependent diabetes mellitus type II gene (Sladek, R. *et al*, 2007).

6.4.2.5 Upper lip drop

One association was found between upper lip drop and rs1610305 ($5.0x10^{-7}$), which is located within 20p13. The nearby gene to this SNP is *STK35*, which encodes the Serine/threonine kinase 35 protein. This kinase protein is predominantly found in the nucleus. The encoded protein may be a regulator of actin stress fibres in non-muscle cells (Goyal, P. *et al*, 2011).

6.4.2.6 Upper lip groove

Upper lip groove was associated with several SNPs located within the 1q42.11-13 locus; the most significant SNP was rs4601553 ($8.3x10^{-7}$). The gene in closest proximity to this SNP is *ZNF678* which encodes the Zinc finger protein 678, which is a transcription factor belonging to the zinc finger binding protein family.

The SNP rs1390401, which is also located within *ZNF678* has been robustly associated with height in a study of 13,665 European individuals, and replicated in a population of 16,482 ($p=5.4x10^{-9}$) (Weedon, M.N. *et al*, 2008) and further validated in a population study of over 170,000 individuals ($p=1.3x10^{-8}$) (Speliotes, E.K. *et al*, 2010, Wood, A.R. *et al*, 2014, Ried, J.S. *et al*, 2016).

This region is also in close proximity to the *LEFTY1* gene, which is located in the 1q42.12 locus; associations with this gene and facial patterning have been previously described (Chapter 2&6).

6.4.3 Lower lip

6.4.3.1 Lower lip fullness

One association was found between lower lip fullness and rs1035055 ($9.4x10^{-7}$), which is located within 2q24.2. The nearby gene to this SNP is *TANC1*, which encodes the Tetratricopeptide protein. This protein may work as a postsynaptic scaffold component by forming a multiprotein complex with various postsynaptic density proteins and have a key role to play in brain morphology (Suzuki, T. *et al*, 2005).

6.4.3.2 Lower lip contour

One association was found between lower lip contour and rs6971502 ($7x10^{-7}$), which is located within 7p14.1. The nearby gene to this SNP is *CDK13*, which encodes the cyclin-dependent kinase 13 enzyme. It is a member of the cyclin-dependent serine/threonine protein kinase family, which plays essential roles as master switches in cell cycle

control (Lim, S. *et al*, 2013). It may also have a role to play in regulating cell viability in prostate cancer (Mo, W. *et al*, 2013).

6.4.3.3 Lower lip vermilion border

One association was found between lower lip vermilion border and the SNP rs10463015 ($5.3x10^{-7}$), which is located within 5q35.1. The nearby gene is *UBTD2*, which encodes the ubiquitin domain containing 2, it is also known as dendritic cell derived ubiquitin-like protein (DCUBP). It has been implicated in apoptosis, cellular differentiation and tumour genesis (Liu, S. *et al*, 2003). It has also been associated with holoprosencephaly, facial dysmorphisms and preaxial polydactyly (Koolen, D.A. *et al*, 2006).

This SNP also arises in close proximity to the *MSX2* gene, which is located at 5q35.2. MSX2 is required for proper craniofacial morphogenesis (Johnston, M.C. *et al*, 1995), and mutations are associated with craniosynostosis type 2 and CL/P (Vieira, A.R. *et al*, 2005).

6.4.3.4 Lower lip double vermilion border

Lower lip double border had three associations; rs6439121 ($1.8x10^{-7}$) located within 3q21.3, rs2955102 ($2.4x10^{-7}$) located within 3q21 and rs5933620 ($7.9x10^{-7}$) located within Xp11.22.

The nearby gene to the SNP rs6439121 is *EEFSEC*, which encodes Eukaryotic elongation factor, selenocysteine-tRNA-specific. It is a translation factor, which is necessary for the incorporation of selenocysteine into proteins. It has been suggested to play a possible role in age at menarche ($P=1.3x10^{-7}$) (Elks, C.E. *et al*, 2010)

The nearby gene to the SNP rs2955102 is *RUVBL1*, which encodes RuvB-like AAA ATPase 1 protein coding. This protein is associated with several transcriptional and protein complexes involved in both ATP-dependent remodeling and histone modification. It plays an essential role in oncogenic transformation by *MYC* (Si, J. *et al*, 2010) and may play a role in tumour suppression and activation (Mayes, K. *et al*, 2014). *MYC* lies within the 8q24 locus, which is a significant cleft risk locus (Beaty, T.H. *et al*, 2013, Beaty, T.H. *et al*, 2016).

The nearby gene to the SNP rs5933620 is *KDM5C*, which encodes lysine demethylase 5C. This protein belongs to the highly conserved ARID protein family. It contains several DNA-binding motifs that link it to transcriptional regulation and chromatin

remodeling regulation (Jensen, L.R. *et al*, 2005). Mutations are a relatively common cause of X-linked intellectual disability (Iwase, S. *et al*, 2007) and might play an important role in human brain function.

6.4.3.5 Lower lip brim

One association was found between lower lip brim and the SNP rs17035178 ($5.1x10^{-7}$), which is located within 2p14. The nearby genes are *CNRIP1* and *PPP3R1*. *CNRIP1 encodes* the calcineurin subunit B type 1 protein.

Mutations associated with the *PPP3R1* gene include multiple synostosis syndromes (Laue, K. *et al*, 2011).

6.4.3.6 Lower lip drop

One association was found between lower lip drop and the SNP rs17629330 ($8.3x10^{-7}$), which is located within 4p15.1. The nearby gene is *LOC105374557*, which is 500kbp away from any gene.

6.4.3.7 Lower lip groove

One association was found between lower lip groove and the SNP rs231361 ($4x10^{-7}$), which is located within 11p15.5. The SNP lies within the gene *KCNQ1*, which encodes the potassium voltage-gated channel subfamily KQT member 1 (Melman, Y.F. *et al*). It plays an important role in cardiac repolarisation and mutations are associated with hereditary long QT syndrome 1 (Romano-Ward syndrome) (Arbour, L. *et al*, 2008), Jervell and Lange-Nielsen syndrome (Qureshi, S.F. *et al*, 2013).

The *KCNQ1* gene has previously been associated with height ($p=2.32x10^{-8}$) (Speliotes, E.K. *et al*, 2010, Wood, A.R. *et al*, 2014, Ried, J.S. *et al*, 2016)

6.4.4 Sub-lip

6.4.4.1 Mentolabial Fold

Mentolabial fold had two associations; rs6061441 ($9.7x10^{-8}$) located within 20q13.3 and rs4820309 ($8.0x10^{-7}$) located within 22q11.22.

The SNP rs6061441 association with mentolabial fold reached the Bonferroni correction. This SNP lies within the *CDH4* gene, which encodes the calcium-dependent cell-cell adhesion glycoprotein cadherin-4. It is thought to play an important role during brain segmentation and neuronal outgrowth, as well as a role in kidney and muscle development. *CDH4* may have a protective role in tumour suppression (Du, C. *et al*, 2011).

Cadherin-4 has also been observed in the myoblastic cells of the mandibular arch, during early embryological development in chick embryos (Mootoosamy, R.C. *et al*, 2002, Nogueira, J.M. *et al*, 2015). 20q13.3 deletion is rare, and can result in intellectual disability, absent speech, hypotonia, pre- and post-natal growth retardation and an abnormal face with a unilateral CL(Butler, M.G. *et al*, 2013).

The SNP rs4820309 located within 22q11.22 lies within the *TOP3B* gene. It encodes the DNA topoisomerase enzyme, which controls and alters the topologic states of DNA during transcription and plays a role in DNA recombination, cellular ageing, and maintenance of genome stability (Yang, Y. *et al*, 2014). Low expression of this gene may be related to higher survival rates in breast cancer patients (Oliveira-Costa, J.P. *et al*, 2010).

Individuals with 22q11.2 deletion (DiGeorge Syndrome) have congenital heart defects, impaired immunity and CP. They also have facial anomalies, including lack of tonicity of the lower lip region and asymmetric crying faces (Digilio, M. *et al*, 2005).

6.4.4.2 Chin dimple

Chin dimple had two associations; rs11017876 ($2.3x10^{-8}$) located within 10q26.13-q26.3 and rs16889815 ($8x10^{-7}$) located within 5p13.3.

The association between the SNP rs11017876 and chin dimple met the stringent Bonferroni correction ($P=2.3x10^{-8}$). This SNP is located within the *DOCK1* gene, which encodes dedicator of cytokinesis protein 1. This protein regulates the small GTPase Rac, thereby influencing several biological processes, including phagocytosis and cell migration. Overexpression of this gene has been associated with certain cancers (Zhao,

F. *et al*, 2011), and has been found to play an essential role in mediating the *HER2* pathway in progressive metastatic breast cancer (Laurin, M. *et al*, 2013).

Individuals with 10q26 deletion syndrome have developmental delay and moderate mental retardation. Physical facial characteristics include microcephaly, broad nasal bridge, with beaked or prominent nose. Triangular, asymmetrical faces, with low set ears. Thin bow-shaped upper lip with micrognathic jaw and convergent strabismus (Mehta, L. *et al*, 1987). The 10q26 locus has also been suggested as a cleft susceptibility region (Leslie, E.J. *et al*, 2016).

The gene *FGFR2* is also located at the 10q26.13 locus, and has been associated with a number of craniosynostosis syndromes and CLP (Reardon, W. *et al*, 1994, Hollway, G.E. *et al*, 1997) as well as having an important role in lip and facial development (Bachler, M. *et al*, 2001, Stanier, P. *et al*, 2012).

The SNP rs16889815 is located within the *ZFR* gene. *ZFR* is a member of the zinc finger RNA binding protein family, which are a diverse family of proteins that serve as mediators, binding DNA and RNA proteins.

Chin dimple has also been previously associated with another zinc finger protein (*ZEB2*) in a sample of individuals using a self-reporting method (Eriksson, N. *et al*, 2012). It has also been observed that the chin dimple is inherited amongst family members, in an autosomal recessive nature (Lebow, M.R. *et al*, 1941).

6.4.4.3 Sub-lip tone

Sub-lip tone was associated with the SNP rs7011739 ($3.3x10^{-7}$), located at 8q24.21. The nearest gene is *FAM84B, which* interacts with catenin alpha-1. It is highly expressed in cardiac muscles and plays a role in epithelial tissue, both at adherens junctions (to mediate the anchorage of actin filaments) and in signalling pathways. FAM84B has been robustly associated with breast carcinoma, colorectal carcinoma, oesophageal squamous cell carcinoma and tumour metastasis (Cheng, C. *et al*, 2016). The locus 8q24.21 has also been previously associated with absolute pitch (Theusch, E. *et al*, 2009)

In addition, it is located near the highly significant NSCL/P region 8q24 (Birnbaum, S. *et al*, 2009, Grant, S.F. *et al*, 2009, Beaty, T.H. *et al*, 2010, Ludwig, K.U. *et al*, 2012).

The locus 8q24.21 has also been previously associated with individuals with a deep bite tendency (p=0.018) (Weaver, C.A., 2014); individuals with a deep bite, would also

have a wide deep concavity phenotype (code 3) sub-lip tone, whereas an open bite is associated with proclined lower incisors and would be more likely to be associated with lack of sub-lip tone (code 0).

6.4.5 Skeletal Pattern

Skeletal pattern had three associations; rs12767587 ($5x10^{-7}$) located within 10p12.1, rs402800 ($7x10^{-7}$) located within 3q26.31 and rs17752647 ($7x10^{-7}$) located within 2q22.1.

The SNP rs402800 is located within the 3q26.31 locus, and is located within the gene region *TNFSF10*. *TNFSF10* is a member of the TNF-ligand family, and is expressed on most cells. It is regulated by cytokines, growth factors, injury, viral infection, chromatin modification, and by a variety of transcription factors (Wood, A.R. *et al*, 2014).

The locus 3q26.2 has been robustly associated with skeletal III patterns amongst Hispanic families through lateral cephalometric measurements and linkage analysis (Frazier-Bowers, S. *et al*, 2009, Da Fontoura, C.S. *et al*, 2015). The 3q26 locus has also been associated with height (p=1.74x10-13) (Speliotes, E.K. *et al*, 2010, Wood, A.R. *et al*, 2014, Ried, J.S. *et al*, 2016).

The SNP rs12767587 is located within the 10p12.1 locus, and is located within the gene region *GPR158*. This is an androgen-regulated gene, which encodes G-protein coupled receptor 158. It is highly expressed in the brain, and is linked to neuroendocrine differentiation (Patel, N. *et al*, 2015). It has also been shown to stimulate cell proliferation in prostate cancer cell lines (Patel, N. *et al*, 2013).

Individuals with deletion at the chromosomal region 10p11.23-p12.1 have characteristic phenotypes with marked midface retrusion (Okamoto, N. *et al*, 2012).

The 10q21 region is also associated with bone density and osteoporosis risk later in life (Ralston, S.H. *et al*, 2005).

The SNP rs17752647 is located within the 2q22.1 locus, and is located within the gene region *NXPH2.* It is also expressed in the brain. Signalling molecules that resemble neuropeptides that act by binding to alpha-neurexins and possible other receptors

Nebulin on chromosome 2q22 (*NEM2*) has been associated with Nemaline myopathy, which is characterised by muscle weakness, predominantly affecting facial and axial muscles, resulting in myopathic faces (Pelin, K. *et al*, 1997).

6.5 Replication phase

In order to validate the findings from the discovery phase of the GWAS, ideally the replication sample should be from the same population group, and should be a large enough population sample for statistical power. Ideally, the images for phenotyping should be captured using the same 3D laser camera, and individuals should have genotyping using comparable methods. Unfortunately, this population sample was not available in order to perform GWAS. There was one dataset available to perform the replication phase, an Australian dataset containing genotype and digital photographs of 3,215 twins and siblings.

6.5.1 Sample

The Australian dataset consisted of 3,215 twins and siblings aged between 16-21years of age (Wright, M.J. *et al*, 2004).

6.5.1.1 Ethical Approval

Ethical approval for this study was obtained from the Queensland Institute of Medical Research Human Research Ethics Committee (QIMR-HREC) and was also approved by Cardiff University Dental School Research Ethics committee for sharing the data: An international collaboration exploring gene expression related to facial features Ref (12/34) (Confirmed 11th February 2013).

6.5.1.2 Phenotyping

The two phenotypes that were tested were mentolabial fold and chin dimple. These were the two features that achieved the Bonferroni corrected statistical significant, and it was accepted that these two features were detectable using digital photographs, however, the mentolabial fold may be exaggerated when viewing a 3D laser image (Figure 5.1).

6.5.2 Results

None of the phenotype/genotype associations tested achieved statistical significance (Table 6.4).

Table 6.4 Results of the discovery phase and replication phase

				Discovery phase (N=3,687)		Replication phase (N=3,215)	
Feature	Locus	SNP	Beta	SE	P-value	SE	P-value
Chin dimple	10q26	rs11017876	0.42	0.077	$2.3x10^{-8}$	0.015	0.59
Mentolabial Fold	20q13.3	rs6061441	-0.3	0.057	$9.7x10^{-8}$	0.027	0.93

6.6 Discussion following discovery and replication

Two of the twenty-five lip phenotypes were found to achieve GWAS statistical significance following the stringent Bonferroni correction in the discovery phase.

The two lip phenotypes (mentolabial fold and chin dimple) were demonstrated to behave in an almost independent manner compared to other lip phenotypes (Chapter 4). This suggests that there may be a single gene affecting these lip phentoypes. Replication was attempted with the two lip phenotypes; however, it was unsuccessful with the Australian photographic data set. This could be due to lack of a true effect in the initial ALSPAC sample (Type 2 error), insufficient sample size, capture method with some of the photographs being of low quality and taken too far away from the subject or population sample in the replication set.

Twenty-nine associations were also discovered, which did not quite meet the stringent Bonferroni correction. Nine of these arose in cleft susceptibility loci (Table 6.5).

The SNP rs10463015 arising in 5q35.1 was found to be associated with lower lip vermilion border ($P=5.3x10^{-7}$) in this ALSPAC sample. This locus has also been associated with normal facial variations (nasion) in a population of 5,388 Caucasians (Liu, F. *et al*, 2012c).

The locus 8q24 has been robustly associated with cleft susceptibility in a number of studies; it has also been associated with normal facial variation (bizygomatic distance P=0.017) (Boehringer, S. *et al*, 2011). In this sample, it was associated with lower lip tone ($P=3.3x10^{-7}$). An association was also observed between lower lip double vermilion border and the gene *RUVBL1* ($P=2.4x10^{-7}$), which is known to interact with gene *MYC*, which is also located at 8q24 (Si, J. *et al*, 2010).

Table 6.5 Discovery GWAS near hits with associations in cleft susceptibility loci

Feature	GWAS results				Cleft susceptibility locus	
	P-Value	Reference SNP	Cytogenetic location	Gene	Cytogenetic location	Gene
Upper lip vermilion border	$8.5x10^{-7}$	rs12693640	2q32.3	*TMEFF2*	2q33.2	*SATB2*
Lower lip double vermilion border	$2.4x10^{-7}$	rs2955102	3q21	*RUVBL1*	8q24	*MYC*
Lower lip vermilion border	$5.3x10^{-7}$	rs10463015	5q35.1	*UBTD2*	5q35.2	*MSX2*
Upper lip double border	$3.0x10^{-7}$	rs2365281	7q36	*RNF32*	7q36.3	*SHH*
Upper lip Fullness	$5.7x10^{-7}$	rs687542	8q22.2	*NCALD*	8q22.2	
Lower lip tone (UP)	$3.3x10^{-7}$	rs7011739	8q24.21	*FAM84B*	8q24	*MYC*
Upper Lip Contour	$8.6x10^{-7}$	rs10820934	9q22.2	*SPTLC1*	9q22	
Chin dimple	$2.3x10^{-8}$	rs11017876	10q26.13	*DOCK1*	10q26	*FGFR2*
Mentolabial fold	$8.0x10^{-7}$	rs4820309	22q11.22	*TOP3B*	22q11.2	

** Interacts with*

6.7 Conclusions

The objective was to:

- Perform a genome-wide association study (GWAS) of lip phenotypes

Two phenotype/genotype associations were found in the discovery sample that reached GWAS significance; *DOCK1* and chin dimple and *CDH4* and mentolabial fold. These two lip features appear not to be associated with other features of the lip region, and behave in an independent manner (Chapter 4).

Several phenotypes had near hit associations with cleft susceptibility loci, suggesting that cleft genes may affect normal variation in lip morphology.

It was only possible to attempt replication with two out of the twenty-five features in the replication phase. Replication was not achieved in the independent sample. This could be due to a number of issues: lack of statistical power, insufficient sample size, measurement error, different facial capture systems, or differing population samples.

Chapter 7: Exploration of the effect of non-syndromic cleft SNPs on lip phenotypes

7.1 Introduction

Attempts have been made to determine how NSCL/P SNPs may affect normal facial morphology (Mossey, P.A. *et al*, 2010, Boehringer, S. *et al*, 2011, Liu, F. *et al*, 2012b, Peng, S. *et al*, 2013, Miller, S.F. *et al*, 2014a). These methods have involved landmarking regions of the face, and subsequent analysis of distances, or PCA. The majority of the successes in prior studies have involved regions attributable to bony landmarks, in particular, those which have good reproducibility (Peng, S. *et al*, 2013). However, little is known as to how these common genetic variants affect the lip region. The majority of genetic studies have been based on congenital craniofacial malformations, in which a genetic incident causes a catastrophic effect (rare mutations).

Some studies have assessed the effect of these common variants on facial morphology. It has been suggested that unaffected siblings of NSCL/P have wider noses and bizygomatic distances compared with controls. In addition, *IRF6* may affect the relative protrusion of the upper lip (Boehringer, S. *et al*, 2011). The lip region, however, has subtle soft tissue variations, which are simply overlooked when using traditional methods.

7.1.1 Objective

The objective was to:

- Assess for associations of non-syndromic cleft lip/palate (NSCL/P) single nucleotide polymorphs (SNPs) to lip phenotypes

7.2 Methodology

7.2.1 Subjects

Facial scans were available for 4,747 15 years olds (2,514 females and 2,233 males).

7.2.2 Lip phenotypes

The lip classification scale (Figure 4.4 and Table 4.2) consists of 25 independent lip phenotypes spanning from the philtrum to the sub-lip area, and the skeletal pattern.

7.2.3 Genetic data

Genetic data was available for 3,687 (1,737 males and 1,950 females) individuals, who were genotyped with either the Illumina 317K or 610K genome-wide SNP genotyping platforms by the Wellcome Trust Sanger Institute (Cambridge, UK) and the Centre National de Genotypage (Evry, France). A common set of SNPs (present in both genotyping platforms) were extracted and the resulting raw genome-wide data were subjected to standard quality control methods (Paternoster, L. *et al*, 2012).

7.2.4 SNP selection and genotyping

Seventeen SNPs that have formerly achieved genome-wide significance for association with NSCL/P (Table 2.3) were assessed for association with various lip phenotypes.

7.2.5 NSCL/P genetic risk score

The unweighted NSCL/P genetic risk score was calculated as the sum of NSCL/P genetic alleles for an individual, using imputed dosage data.

7.2.6 Statistical analysis

The genetic risk scores were tested for association with each of the 25 lip phenotypes, in addition, each SNP was also tested individually in a secondary analysis. Statistical analysis was performed using STATA software. Logistical, ordinal or multinomial regression analysis was performed according to whether the lip phenotype outcome was binary, ordered- categorical or unordered-categorical.

Table 7.1 Statistical analysis

Data	Statistical test	Lip Phenotype	Units
Binary	Logistic Regression (logistic)	Upper lip border; Double border; Groove; Drop; Brim; Mentolabial fold; Chin dimple	OR
Ordered Categorical	Ordinal Regression (Ologit)	Philtrum width; Cupid's bow shape; NLA; Lip Fullness; Upper lip contour; Lower lip border; Lower lip contour; Commissures; Lip-chin shape; Tone; Tone up; skeletal pattern	OR
Unordered Categorical	Multinomial Regression (Mlogit)	Philtrum shape	RRR

Multi-testing was taken into account in assessing strength of statistical significance. As 25 phenotypes were tested, an alpha value of 0.002 was used to determine associated variables (0.05/25).

7.3 Results

The average NSCL/P genetic allele score for the 3,687 ALSPAC individuals was 12.58 (95% CI 12.49-12.66), 12.62 in males and 12.53 in females. NSCL/P genetic allele scores varied from 3.73 to 21.93. Individuals with a higher NSCL/P genetic allele score would carry more susceptibility to cleft risk.

7.3.1 NSCL/P genetic allele scores

Given the low available power to detect associations with individual SNPs, a combined NSCL/P genetic risk score was generated from all the NSCL/P variants, and tested for association with the lip phenotypes (Table 7.2).

Table 7.2 Logistic regression results of phenotypes associated with the NSCL/P genetic risk score

Trait	NSCL/P genetic allele effect	Odds Ratio	Confidence Interval		P-Value
			Lower	Upper	
Cupid's bow shape	V-shaped	1.05	1.02	1.07	0.0002*
Philtrum width	Narrow philtrum	1.05	1.02	1.07	0.0003*
Commissures	Upturned	1.03	1.003	1.05	0.03
Philtrum shape	Deep groove	1.071	1.014	1.13	0.045

* Meets Bonferroni corrected alpha threshold (P=0.002)

Two associations met a Bonferroni corrected p-value (P=0.002), and two associations showed nominal associations. All the increased odds ratios are small, highlighting the low penetrance nature associated with these common variants.

Statistically significant associations were observed with a V-shaped Cupid's bow (P=0.0002) and a narrow philtrum width (P=0.0003) showing small increased odds ratios. Nominal association was observed with an upturned commissure (P=0.03) and deep groove philtrum shape (P=0.045).

7.3.1.1 V-shaped Cupid's bow shape

A V-shaped Cupid's bow was associated with an increased odds ratio of 1.05 (P=0.0002), and a mean NSCL/P genetic risk score of 12.78, with a range (12.65-12.92), whilst a U-shaped Cupid's bow shape had a mean NSCL/P genetic risk score of 12.43, with a range (12.32-12.54) (P=0.0003). A Flat Cupid's bow had a higher mean NSCL/P genetic risk score than U-shaped (12.52, range 12.20-12.84). Indicating that individuals with a V-shaped Cupid's bow posses a higher number of NSCL/P genetic alleles compared to those with a U-shaped Cupid's bow.

This can be further explored by assessing NSCL/P genetic allele scores for Cupid's bow shape according to sex. This reveals that for males, a flat Cupid's bow shape (12.71, 12.17-13.24) carries a similar risk to a V-shaped Cupid's bow (12.86, 12.66 – 13.07). Whilst for females, it is only a V-shaped Cupid's bow, which carries a higher risk (12.73, 12.56-12.90) (Figure 7.1).

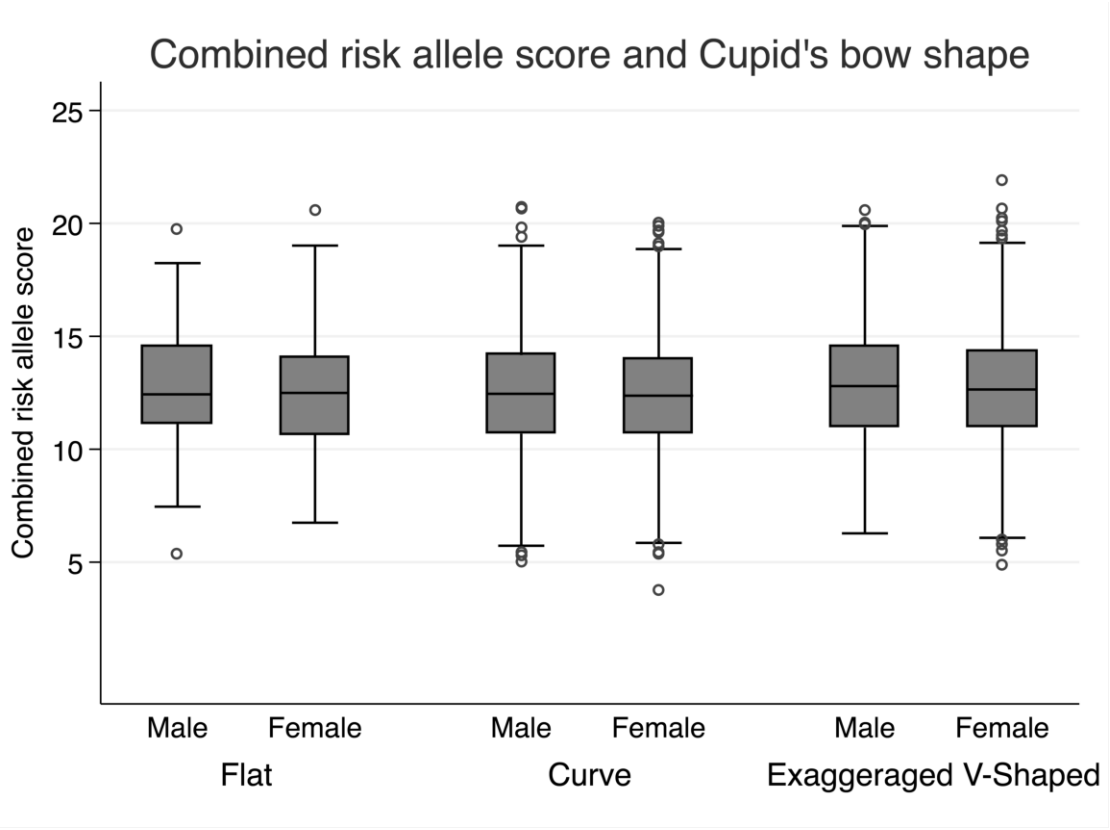


Figure 7.1 Box plot graph displaying the distribution of NSCL/P genetic alleles according to Cupid's bow shape and sex

7.3.1.2 Narrow philtrum

A narrow philtrum was associated with an increased odds ratio of 1.05 (P=0.0003), and a mean NSCL/P genetic risk score of 13.01, with a range (12.77-13.25), whilst a wide philtrum mean NSCL/P genetic risk score was 12.40, with a range (12.21-12.58). Indicating that individuals with narrow philtrum posses a higher number of NSCL/P genetic alleles compared to those with wide philtrum.

This can be further explored by dividing philtrum width according to sex (Figure 7.2). Males appear to be driving the higher NSCL/P genetic allele score for narrow philtrum width (13.32, 12.88-13.76), compared with females (12.86, 12.58-13.15).

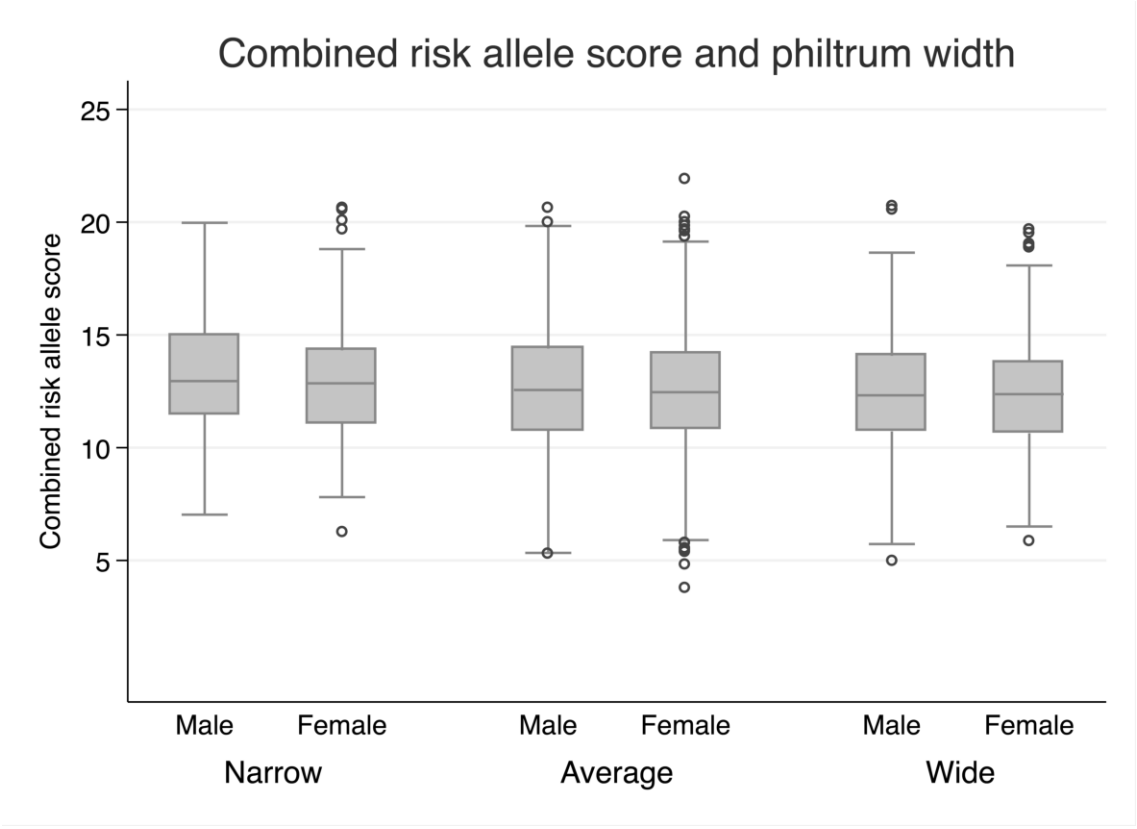


Figure 7.2 Box plot graph displaying the distribution of NSCL/P genetic alleles according to philtrum width and sex

7.3.3 Associated features and genes

Given that four phenotypes were associated with overall risk score, further analysis was necessary, to test whether this was driven by a particular SNP. Although of limited power, each of the each of the seventeen NSCL/P SNPs was tested individually with the twenty-five phenotypes (Table 7.3). Three of the associations tested met a Bonferroni correction for number of phenotypes tested, narrow philtrum ($P=2.2x10^{-3}$), v-shaped Cupid's bow shape ($2.48x10^{-3}$) and skeletal II pattern ($1.58x10^{-6}$).

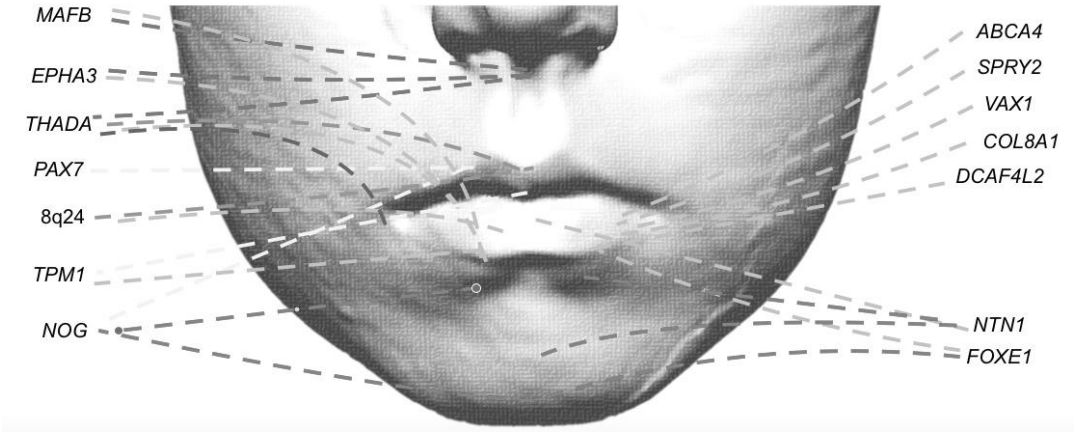


Figure 7.3 Effect of NSCL/P SNPs on lip phenotypes
Red: Philtrum, Orange: Cupid's bow, Yellow: Upper lip, Green: Lower lip, Blue: Commissures, Purple: Sub-lip

Table 7.3 Effect of individual NSCL/P SNPs on lip phenotypes

Genetic Variants		Feature	Effect	OR	P-Value
rs560426	*ABCA4*	Lower lip	No drop	1.15	$<3.7x10^{-3}$
rs742071	*PAX7*	Upper lip	No border	1.17	<0.03
rs7590268	*THADA*	Philtrum width	Narrow	1.17	$<2.2x10^{-3}$
		Cupid's bow	V-shaped	1.15	$<9.9x10^{-3}$
		Lower lip	Full border	1.39	<0.03
		Commissures	Upturned	1.11	<0.03
rs7632427	*EPHA3*	Philtrum Shape	Deep	1.56	$<3x10^{-3}$
			Indentation nose	1.16	<0.05
		Lower lip	No border	1.24	<0.02
rs793464	*COL8A1*		No brim	1.10	<0.03
rs12543318	*DCAF4L2*		Flat contour	1.12	$<5.24x10^{-3}$
rs987525	*8q24*		Thin	1.21	<0.02
		Cupid's bow	V-shaped	1.18	$<2.48x10^{-3}$
rs6478391	*FOXE1*	Skeletal	Skeletal II	1.13	<0.04
		Lower lip	No border	1.26	<0.05
rs4752028	*VAX1*		Double border	1.14	<0.04
rs8001641	*SPRY2*		No brim	1.10	<0.02
rs1873147	*TPM1*		Flat contour	1.11	<0.03
		Upper lip	Groove	1.17	<0.01
rs11650357	*NTN1*	Chin dimple	Dimple	1.15	<0.03
		Lower lip	Groove	1.16	<0.02
rs1880646		Lip-Chin shape	Angular with bumping	1.11	<0.04
rs227731	*NOG*	Skeletal	Skeletal II	1.21	$<1.58x10^{-6}$
		Lip-chin shape	Angular with bumping	1.10	<0.03
		Lower lip tone		1.11	<0.04
		Upper lip	No drop	1.12	$<5.4x10^{-3}$
			Double border	1.27	<0.02
rs13041247	*MAFB*	Philtrum width	Narrow	1.13	<0.02
		Lower lip	Drop	1.13	<0.03
			Full border	1.30	<0.04
rs6029326			Flat contour	1.10	<0.03

Associations reaching nominal association ($p<0.05$) between the NSCL/P SNPs and lip phenotype. Bonferroni-corrected significance threshold is $p<2.1x10^{-3}$.

7.3.3.1 Philtrum

Three SNP regions appeared to affect the philtrum region: rs13041247 (*MAFB),* rs7632427 (*EPHA3)* and rs7590268 *(THADA)* (Figure 7.3). A narrow philtrum width was significantly associated with the generated NSCL/P genetic allele score (Table 7.2), and logistic regression suggests that it may be driven by SNP rs7590268 ($P=2.2x10^{-3}$) in particular (Figure 7.4).

The SNP rs13041247 was also associated with a narrow philtrum (P=0.019).

Two SNPs were associated with Cupid's bow shape were rs7590268 *(THADA)* and rs987525 (8q24) (Figure 7.3). The association with rs987525 ($2.48x10^{-3}$) met the Bonferroni correction, and it may be this SNPS in particular that is driving the NSCL/P genetic allele score (Table 7.2).

7.3.3.2 Upper lip

Three SNP regions appeared to be associated with the upper lip; *rs227731 (NOG)* and *rs742071* (*PAX7*) were associated with the vermilion border, and rs1873147 (*TPM1*) showed an association with upper lip groove (Figure 7.3). None of these associations met the Bonferroni correction.

7.3.3.3 Lower lip

Thirteen SNPs showed association with features of the lower lip; of those, five were associated with the lower lip vermilion. SNP rs13041247 (*MAFB*) and rs7590268 (*THADA*) were associated with a full vermilion border, whilst rs6478391 (*FOXE1*) and rs7632427 (*EPHA3*) were associated with lack of vermilion border (Figure 7.3). In addition, rs4752028 (*VAX1*) was associated with lack of double vermilion border (P=0.04).

7.3.3.4 Sub lip region

Three SNP regions appeared to affect the sub-lip region: rs227731, rs6478391 and rs1880646 (Figure 7.3). The SNP rs1880646 (*NTN1*) was associated with relative indentation of the features of the lower lip and sub-lip region, at the midline, appearing to induce a lower lip groove, chin dimple and angular lip-chin shape with bumping. SNP rs227731 was also associated with lower tone that was angular with bumping (P=0.03).

7.3.3.5 Skeletal pattern

The most significant association was observed with skeletal pattern and rs227731 (*NOG*) (P=1.58x10^{-6}) and also a nominal association was observed with rs6478391 (*FOXE1*) (P=0.036). In total, 2,332 skeletal II, 954 skeletal I and 401 skeletal III patterns were observed amongst this population. The NSCL/P genetic alleles for both associations suggested that a skeletal II pattern (relative retrusion of the lower jaw) is associated with the NSCL/P risk variant.

7.3.4 Sex-stratified variations

Chapter 5 demonstrated that many of the less common lip phenotypes show a sex predilection. Multivariable logistic regression was performed to test whether the NSCL/P genetic allele score was affected by sex, i.e. whether some of the risk traits are sex specific (Table 7.4).

Table 7.4 Variations in mean NSCL/P allele scores stratified by sex

Trait		**Common Variant Effect**	**Mean NSCL/P allele score**	**Mean NSCL/P allele score for opposite sex**	**P-Value**
Philtrum Shape	Female	Indentation near vermilion border	12.85	12.65	<0.00005
		Deep groove into VB	12.84	12.55	
	Male	Deep groove	13.04	12.81	
Philtrum width	Male	Narrow	13.32	12.86	<0.00005
Cupid's bow	Male	Flat	12.71	12.42	<0.00005
Upper lip vermilion border	Male	Absent	12.75	12.54	<0.00005
Upper lip double vermilion border	Female	Present	12.81	12.43	<0.00005
Upper lip drop	Male	Present	12.70	12.48	<0.00005
Lower lip border	Female	Absent	12.73	12.37	<0.00005
Lower lip double border	Males	Absent	12.67	12.48	<0.03
Lower lip brim	Male	Absent	12.66	12.47	<0.001
Lower lip groove	Female	Present	12.79	12.57	<0.004
Lower lip drop	Male	Present	12.61	12.37	<0.00005
Commissures	Female	Upturned	12.90	12.58	<0.00005
Lip-chin shape	Female	Angular	12.77	12.45	<0.003
Tone	Male	Flat	12.69	12.46	<0.05
Tone-up	Female	Angular with bumping	12.83	12.65	<0.003

	Male	Smooth	12.60	12.40	
Skeletal	Female	Skeletal II	12.68	12.58	<0.00005
	Male	Skeletal III	12.79	12.34	

The multivariable logistic regression demonstrated that NSCL/P genetic alleles are sex specific. Amongst females, a higher NSCL/P genetic allele score was observed with a philtrum shape with Indentation near vermilion border, upper lip double vermilion border, absent lower lip border, Lower lip groove, angular lip-chin shape, upturned commissures, angular with bumping tone-up and skeletal II pattern.

Whilst amongst males there were many more phenotypes that carried an increased NSCL/P genetic allele score compared with female, in particular, a flat Cupid's bow, absent upper lip vermilion border, an upper or lower lip drop and a skeletal III pattern.

7.4 Discussion

NSCL/P SNPs appear to affect certain features of the lips and surrounding region. The upper lip phenotypes are narrow philtrum, V-shaped Cupid's bow, and absence of drop, border, and presence of double border. The lower lip phenotypes are curved contour, thin lip, and absence of vermilion border, drop and double border. The sub-lip phenotypes are skeletal II pattern, marked angular lower lip-chin shape and marked lower lip tone.

7.4.1 Philtrum

A narrow philtrum width was associated with the combined NSCL/P genetic allele score (p=0.0003), and in addition, surfaced during single-SNP analysis. Two variants were suggestive of having a role to play in determining a narrow philtrum: *rs7590268* (P=0.002) and *rs13041247* (P=0.019). The SNP *rs13041247* maps 45kb downstream of the musculoaponeurotic fibrosarcoma oncogene homolog B (*MAFB*) gene, which encodes the v-maf transcription factor. The exact role that this SNP plays in the manifestation of NSCL/P has not yet been described, however, animal studies have indicated its expression within craniofacial structures during embryogenesis (Beaty, T.H. *et al*, 2010). It has also has been associated with mouth width amongst individuals with NSCL/P (Miller, S.F. *et al*, 2014a).

A deep philtrum shape was also associated with the combined NSCL/P genetic allele score (P=0.045) and arose during the single SNP analysis with the SNP rs7632427 ($P=3x10^{-3}$). This SNP is located approximately 3kb downstream of the *EPHA3* gene, this family of genes are involved in the regulation of cell shape and cell-cell contacts (Saha, N. *et al*, 2007).

7.4.2 Cupids bow

A v-shaped Cupid's bow was associated with the combined NSCL/P genetic allele score (P=0.0002*), and also arose during the single SNP analysis. The two SNPs rs7590268 ($P=9.9x10^{-3}$) and rs987525 $P=2.48x10^{-3}$) are suggestive of playing a role in the development of a v-shaped Cupid's bow. The SNP rs987525 has been robustly associated with NSCL/P achieving large statistically significant p-values ($P=5.12 \times 10^{-35}$) (Ludwig, K.U. *et al*, 2012) ($P=3.34 \times 10^{-24}$) (Birnbaum, S. *et al*, 2009)

SNP rs987525 maps to an intergenic region of 8q24, which may control expression of the proto-oncogene *MYC* in the developing murine facial prominences (Uslu, V.V. *et al*, 2014). Deletion of this protein leads to mild alterations in facial morphology in mice, and sporadically leads to CL/P.

7.4.3 Skeletal Pattern

An association was found between rs227731 and skeletal II pattern (mandibular retrognathia, $P=1.58x10^{-6}$). This SNP is located 100kb centromeres from *NOG* gene, which encodes for the noggin protein (Mangold, E. *et al*, 2010, Ludwig, K.U. *et al*, 2012). A relationship between *NOG* and skeletal II pattern has been previously postulated by several authors, but has so far failed to demonstrate a convincing association (Gutierrez, S.J. *et al*, 2010, Da Fontoura, C.S. *et al*, 2015). However, a previous study identified that parents of children with CP were more likely to have class II malocclusions (as a result of mandibular retrusion) (Prochazkova, J. *et al*, 1986).

NOG encodes the noggin protein, which is an antagonist of members of the transforming growth factor beta (*TGF-ß*) superfamily, which includes bone morphogenetic protein 4 (*BMP4*). *BMP4* has been shown to regulate palatogenesis (Zhang, T.X. *et al*, 2012) and many animal studies have demonstrated that they play an important role in regulating a diverse number of developmental processes and the evolution of facial shape and size (Wan, M. *et al*, 2005).

The Bmp pathway plays an important role during lip development (Chapter 2 and Table 2.1). *Bmp4* is highly expressed in the distal epithelia of the medial and lateral nasal, maxillary and mandibular processes (Wan, M. *et al*, 2005).

Ectopic application of either *Bmp2* or *Bmp4* proteins induces overgrowth and produces changes to the patterning of the facial primordia (Barlow, A.J. *et al*, 1997). Inhibiting *Bmp* signaling by application of *Noggin* causes reduced mesenchymal proliferation and outgrowth (Ashique, A.M. *et al*, 2002). In addition, research involving mutant mice has also shown that mice lacking the *Nog* gene have enlarged mandibles (Matsui, M. *et al*, 2014). It is hypothesised that this manifests due to an increase in size of Meckel's cartilage during mandibular development, thus proposing that lack of *Nog* results in a prognathic mandible (skeletal III pattern) and an increase in *Nog* produces mandibular hypoplasia (skeletal II pattern).

7.5 Conclusion

The objective was to:

- Assess for associations of non-syndromic cleft lip/palate (NSCL/P) single nucleotide polymorphs (SNPs) to lip phenotypes

A combined score of the NSCL/P genetic alleles suggests that NSCL/P SNPs affect the philtral area and commissures, eliciting a narrow, deep philtrum, V-shaped Cupid's bow and upturned commissures.

Fourteen of the seventeen SNPs reached nominal association ($p<0.05$) with several lip phenotypes. The most significant SNP association was between rs227731 and skeletal II pattern (OR 0.79, $P=1.58x10^{-6}$), indicating relative mandibular retrognathia as the phenotype. Several other variants showed nominal association with various lip phenotypes and may warrant additional replication in future studies, but did not meet our stringent Bonferroni corrected threshold for significance.

THADA and EPHA3 may have a role to play in the development of the upper lip, whilst *NTN1* may have a role in the development of features at the midline of the lower lip.

NSCL/P SNPs appear to affect lip phenotypes, which has not been previously acknowledged. Using this principle, it is worth exploring the notion that unaffected individuals with certain lip phenotypes could be carrying NSCLP NSCL/P genetic alleles. As such, certain phenotypes, such as individuals with mandibular retrognathia have a 2% higher risk of having cleft offspring, compared with those not displaying the lip phenotype.

Chapter 8: Exploration of the prevalence of lip phenotypes amongst case-parent trios

8.1 Introduction

Many studies have suggested that parents of cleft children have altered facial phenotypes compared with parents with no history of OFC (Chapter 2, Table 2.6). These alterations can range from defects in the orbicularis oris muscle (Neiswanger, K. *et al*, 2007), alterations in dental anomalies or malocclusions (Prochazkova, J. *et al*, 1986) to variations in craniofacial skeletal disproportions (Mossey, P.A. *et al*, 1997, Yoon, Y.J. *et al*, 2004, Weinberg, S.M. *et al*, 2009),

The majority of research has been constructed using cephalometric measurements (Mossey, P.A. *et al*, 1997) or using facial landmark measurements or principal components from 3D scans to assess for variation (Weinberg, S.M. *et al*, 2008b, Weinberg, S.M. *et al*, 2009). Previously, research has not involved assessing differences in characteristic features of the lip region. In this study lip phenotypes (Figure 4.4 and Table 4.2) were assessed for prevalence amongst parents of cleft children, and compared with control parents.

The results of the previous analysis for associations between candidate SNPs and lip phenotype suggest that NSCL/P SNPs may affect normal variation. This may have clinical uses if it were possible to predict whether a parent with certain lip phenotypes has an increased risk of having a NSCL/P offspring.

Currently there is no definitive tool for assessing the risk of having a NSCL/P offspring, and it is usually diagnosed prenatally in expectant mothers using ultrasound assessment or at birth. Better understanding of the genetic inheritance of craniofacial features associated with CL/P may contribute to the development of cleft risk assessment methods (Yoon, Y.J. *et al*, 2004).

8.1.1 Objectives

The objectives were to:

- Perform a case/control study to assess the prevalence of lip phenotypes amongst unaffected biological parents of cleft and non-cleft children
- Assess the predictive capability of lip phenotypes as a precursor of cleft risk

8.2 Methodology

The FaceBase consortium agreed to share 597 3dMD images of parents with cleft and non-cleft children (trios) to allow the exploration of lip associations.

In order to standardise the conditions of assessment of the lips, the images were initially landmarked and registered on a standardised 3D reference frame with consistent lighting. From these 3D images, six 2D standardised photographs were created (Figure 4.2) to allow the assessment of the lips from different perspectives. The images were captured using colour texture, as this was found to be most favourable for assessing lip phenotypes, and drawing comparisons from laser images (Table 5.2). It was possible to classify 23 out of the 25 lip phenotypes using the modified classification tool.

The images were randomised so that the assessor was blind to cleft and non-cleft parents. There were no personal identifiers but there were unique case identifiers and codes to identify parental pairings and cleft/non-cleft offspring. All lip phenotypes, except upper and lower brim were assessed for prevalence amongst the two groups.

Statistical analysis involved logistic regression for parents with cleft and those without, to investigate associations between lip phenotypes and cleft outcome.

8.2.1 Subjects

The FaceBase consortium provided 597 images of case-parent trios (286) and (311) controls captured using 3dMD.

8.2.2 Ethical approval

Ethical approval was obtained from Cardiff University's in house ethics committee and the University of Pittsburgh's. Consent was previously obtained by the University of Pittsburgh, prior to attaining the 3D images.

8.3 Results

A total of 286 case parents (174 mothers and 112 fathers) and 311 control parents (212 mothers and 99 fathers) were available for assessment. Nine features showed a statistically significant ($p<0.05$) difference between case and controls, and an additional four when sex was a covariate (Table 8.1).

8.3.1. Prevalence of phenotypes

The only feature within the philtrum region that had a statistically significant difference between the case/control groups was philtrum width. The control group had significantly greater number of average philtrum width compared with the case group (P=0.042). In addition, mothers in the case group had an increased prevalence of

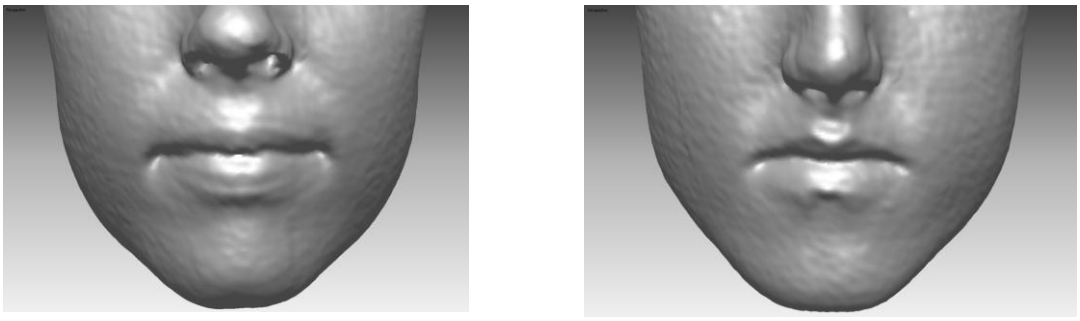


Figure 8.1 Example of features with increasde prevalence amongst cleft parents
Mother (Left) - Narrow philtrum, upper lip groove, convex upper lip contour.
Father (Right) – Thin upper lip, absent lower lip border

narrow philtrum (P=0.001) (Figure 8.1).

Statistically significant differences were observed with several features of the upper lip, including fullness, contour, drop and groove.

Fathers in the case group had an increased prevalence of thin upper lips (P=0.032). The case group overall had an increased prevalence of convex upper lip contour (P=0.018). Control fathers had a higher incidence of pseudo-convex upper lip contour compared with case fathers (P=0.02). Presence of an upper lip groove was also higher amongst the case group (P=0.02), but was only statistically significant amongst mothers.

Features of the lower lip were contour, border, double border and groove. Control parents had an increased prevalence of marked curve lower lip contour (P=0.014),

whereas cleft parents had increased prevalence of flat (1) or narrow at midline (0) lower lip contours (Figure 8.1).

Table 8.1 Prevalence of features amongst case and control parents

Feature	Classification	Control Parents						Case Parents						Chi2 P-values
		Mother		Father		Total		Mother		Father		Total		
		(212)	%	(99)	%	(311)	%	(174)	%	(112)	%	(286)	%	
Philtrum Shape	Smooth	17	8	6	8	23	8	23	14	0	0	23	9	
	Normal gradient	61	30	10	13	71	25	42	25	11	14	53	21	
	Indentation near nose	18	9	2	3	20	7	22	13	1	1	23	9	
	Indentation in middle	53	26	26	33	79	28	38	23	24	30	62	25	
	Indentation near VB	31	15	18	23	49	17	17	10	18	23	35	14	
	Deep indentation	25	12	13	16	38	13	22	13	19	24	41	17	
	Deep into VB	1	<1	4	5	5	2	4	2	7	9	11	4	
Philtrum Width	Narrow	55	27	9	10	64	22	63	38*	13	13	76	29	0.04
	Average	140	68	51	57	191	65*	84	50	60	61	144	54	0.001 (F)
	Wide	12	6	29	33	41	14	21	13*	25	26	46	17	
Cupid's Bow	Flat	13	6	4	4	17	6	18	11	3	3	21	8	
	U - Shaped	79	38	36	40	115	39	60	36	44	44	104	39	
	V - Shaped	115	56	49	55	164	55	90	54	52	53	142	53	
Nasolabial Angle	Acute	24	12	9	10	33	11	18	11	9	9	27	10	
	Normal	131	63	48	53	179	60	115	69	64	64	179	67	
	Obtuse	52	25	34	37	86	29	34	20	27	27	61	23	
Upper lip														
Fullness	Thin	13	6	7	8	20	7	11	7	14	14*	25	9	0.03 (M)
	Medium	151	73	70	77	221	74	122	73	79	81	201	76	
	Thick	43	21	14	15	57	19	34	20	5	5	39	15	
Contour	Concave	43	21	18	20	61	21	18	11	23	24	41	15	0.02
	Straight	97	47	41	47	138	47	85	51	47	48	132	50	0.03 (F)
	Convex	48	23	15	17	63	21	53	32*	24	25	77	29*	0.02 (M)
	Pseudo-convex	18	9	14	16*	32	11	12	7	3	3	15	6	
Border	None	24	12	8	9	32	11	30	18	13	14	4	16	
	Middle only	22	11	11	13	33	11	11	7	18	19	29	11	
	Full border	161	78	69	78	230	78	127	76	63	67	190	73	
Double Border	None	153	74	75	88	228	78	128	76	88	95	216	83	
	Present	54	26	10	12	64	22	40	24	5	5	45	17	
Drop	None	88	43	41	46	129	44	83	50	54	56	137	52*	0.04
	Midline drop	118	57	48	54	166	56	82	50	43	44	125	48	
Groove	None	194	94	84	94	278	94	144	87	92	95	236	90	0.02 (F)
	Groove	12	6	5	6	17	6	21	13*	5	5	26	10	
Lower lip														
Fullness	Thin	7	3	7	8	14	5	7	4	18	18	25	9	
	Medium	156	75	73	80	229	77	130	78	71	72	201	76	
	Thick	44	21	11	12	55	18	30	18	10	10	40	15	
Contour	Narrow at midline	35	17	32	35	67	22	31	19	43	43*	74	28*	0.005
	Straight	13	6	11	12	24	8	21	13	21	21*	42	16*	0.02(M)
	Gentle Curve	136	66	33	36	169	57	97	58	31	31	128	48	
	Curved	23	11	15	16	38	13	18	11	5	5	23	9	
Border	None	16	8	11	12	27	9	19	11	25	25*	44	17*	0.01
	Middle	30	14	16	18	46	15	25	15	22	22	47	18	0.03(M)
	Full	161	78	64	70	225	76	122	73	53	53	175	66	
Double Border	None	47	23	35	38	82	28	50	30	59	58*	109	41*	0.001
	Present	160	77	56	62	216	72	116	70	42	42	158	59	0.006(M)
Groove	None	181	87	76	85	257	87	159	95*	91	92	250	94*	0.004
	Groove	26	13	13	15	39	13	8	5	8	8	16	6	0.009(F)
Bump	None	153	74	64	72	217	73	112	67	69	70	181	68	
	Bump	54	26	25	28	79	27	55	33	30	30	85	32	
Commissure	Upturned	6	3	8	9	14	5	8	5	4	4	12	4	
	Flat	29	14	14	15	43	14	20	12	12	12	32	12	
	Downturned	172	83	70	76	242	81	140	83	84	84	224	84	
Sub-lip														
Lip - Chin Shape	Flat	15	7	9	10	24	8	10	6	7	7	17	6	
	Convex	136	66	37	42	173	58	111	66	44	45	155	58	
	Angular	47	23	38	43	85	29	35	21	38	39	73	27	
	Angular with pronounced vermilion	3	1	2	2	5	2	8	5	3	3	11	4	
	Angular with bump	6	3	3	3	9	3	4	2	6	6	10	4	
Mentolabial Fold	None	104	50	33	37	137	46	77	46	43	43	120	45	
	Present	105	50	57	63	162	54	91	54	56	57	147	55	
Chin Dimple	None	156	76	52	68	208	74	121	75	54	66	175	72	
	Present	49	24	25	32	74	26	41	25	28	34	69	28	
Tone	None	27	13	15	19	42	15	30	18	15	16	45	17	0.001
	Slight convex	73	35	18	23	91	32	47	28	17	19	64	25	0.006(F)
	Square shaped convex	69	33	22	28	91	32	61	36	25	27	86	33	
	Deep convex	27	13	15	19	42	15	9	5	13	14	22	8	
	Bilateral bumping	11	5	9	11	20	7	21	13*	21	23	42	16*	
Tone - Up	None	27	13	15	19	42	15	30	18	15	16	45	17	0.002
	Slight with bump	73	35	18	23	91	32	48	29	17	19	65	25	0.01(F)
	Square shaped convex	69	33	22	28	91	32	59	35	25	27	84	32	
	Deep convex	27	13	15	19	42	15	10	6	13	14	23	9	
	Bilateral bumping	11	5	9	11	20	7	21	13*	21	23	42	16*	
Skeletal Pattern	Skeletal I	95	46	29	32	136	46	97	58	39	39	99	37	
	Skeletal II	86	42	50	55	124	42	55	33	44	44	136	51	
	Skeletal III	26	13	12	13	38	13	16	10	17	17	33	12	

8.3.2 Logistic regression of the prevalence of lip phenotypes amongst cleft parents compared with controls

Logistic regression was performed to assess whether any of the phenotypes were associated with increased cleft risk. The results demonstrated that eight features had increased odds ratios amongst the case parents (Table 8.2).

Table 8.2 Logistic regression of prevalence of lip phenotypes amongst cleft parents compared with controls

Phenotype	Feature	Odds Ratio	Confidence Interval		P-Value
			Lower	Upper	
Philtrum Width	Average	0.65	0.46	0.91	0.012
Upper lip contour	Convex	1.50	1.02	2.21	0.038
Upper lip drop	No drop	1.41	1.01	1.97	0.044
Lower lip border	Absent	1.99	1.19	3.32	0.008
Lower double border	Absent	1.82	1.28	2.58	0.001
Lower lip groove	Absent	2.37	1.29	4.35	0.005
Lower lip contour	Flat	2.13	1.25	3.63	0.005
Lower lip tone / Tone up	Bilateral bumping	2.57	1.467	4.51	0.001
Skeletal	Skeletal I	1.45	1.04	2.02	0.03

The most significant phenotypes were lower lip tone with bumping OR 2.57 (P=0.001), and an absent lower lip double border, (OR 1.82, P=0.001).

An average philtrum width was associated with reduced odds ratio (OR 0.65, P=0.01).

Case parents had increased prevalence of absent lower lip border (P=0.008) and double border (P=0.001); they were also less likely to have a lower lip groove (P=0.005). An angular lower lip tone with bumping (P=0.001) was significantly higher amongst the case group, with 16% of parents with a cleft child having this phenotype, compared with only 7% of controls. Control mothers had a higher incidence of angular tone with brim compared to the case group (P=0.021).

8.3.3 Multivariable logistic regression

Multivariable logistic regression was performed to assess whether any of the associations were driven by sex.

There were nine phenotypes that showed sex predilection amongst case parents. The majority of these were associated with phenotypes of the mother.

Case mothers had an increased prevalence of narrow philtrum (P=0.012), convex upper lip contours (P=0.03), upper lip groove (P=0.05), absent lower lip groove (P=0.004), flat lower lip contour (P=0.009) and angular lower lip tone with bumping (P=0.002).

Case fathers on the other hand, had increased prevalence of absent lower lip border (P=0.015) and lower lip double borders (P=0.003) (Figure 8.2 and Table 8.3).

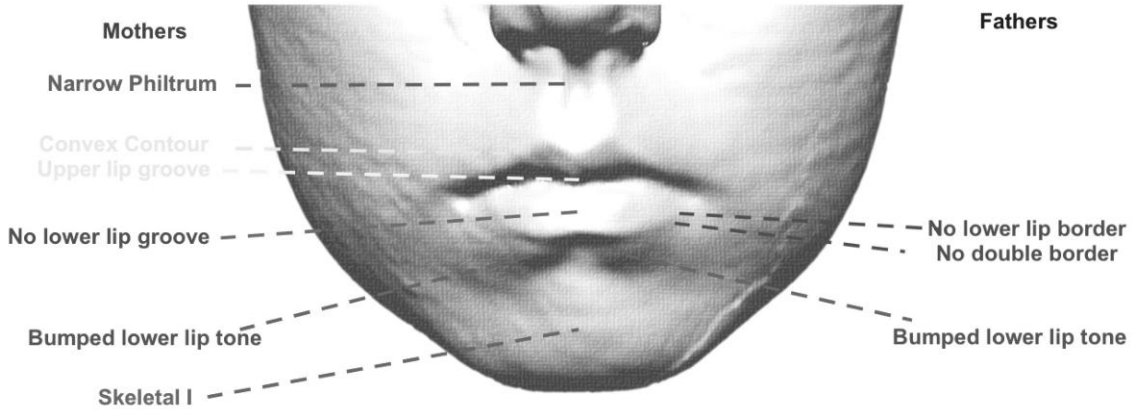


Figure 8.2 Lip phenotypes amongst case parents

Table 8.3 Multivariable logistic regression of prevalence of lip phenotypes amongst cleft parents compared with controls

Phenotype	Feature	Parent	Adjusted Odds Ratio	Confidence Interval		P-Value
				Lower	Upper	
Philtrum Width	Narrow	Mother	0.65	0.46	0.91	0.012
Upper lip contour	Convex	Mother	1.54	1.04	2.27	0.029
Upper lip groove	Present	Mother	1.87	1.00	3.55	0.05
Lower lip border	Absent	Father	1.90	1.13	3.18	0.015
Lower lip groove	Absent	Mother	2.43	1.32	4.47	0.004
Lower lip double border	Absent	Father	1.74	1.21	2.49	0.003
Lower lip contour	Flat	Mother	2.05	1.20	3.50	0.009
Lower lip tone	Angular with bumping	Mother	2.45	1.39	4.32	0.002
Skeletal	Skeletal I	Mother	1.53	1.09	2.14	0.014

8.4 Discussion

There has been a suggestion that unaffected siblings of NSCL/P individuals have increased prevalence of certain facial phenotypes compared to the general population (Mossey, P.A. *et al*, 1997, Yoon, Y.J. *et al*, 2004, Neiswanger, K. *et al*, 2007, Weinberg, S.M. *et al*, 2008b, Weinberg, S.M. *et al*, 2009, Boehringer, S. *et al*, 2011, Defay, D.K., 2011, Miller, S.F. *et al*, 2014a, Leslie, E.J. *et al*, 2016). This study has involved assessing for differences in the prevalence of lip phenotypes (Figure 4.4 and Table 4.2) amongst parents of cleft children compared with control parents.

8.4.1 Phenotypes associated with increased prevalence amongst cleft parents

Logistic regression was performed to assess whether any of the phenotypes were associated with an increased risk of having a cleft offspring. The most significant phenotypes were lower lip tone with bumping OR 2.57 (P=0.001), and an absent lower lip double border, (OR 1.82, P=0.001).

An angular lower lip tone with bumping (P=0.001) was significantly higher amongst the case parents (16%), compared with only 7% of controls. The results of the effect of NSCL/P SNPs on normal variation amongst the ALSPAC cohort also demonstrated that two SNPs were associated with angular tone with bumping; SNPs rs1880646 (*NTN1*) (P=0.03) and rs227731 (*NOG*) (P=0.03).

It was also observed that control parents had a higher incidence of average philtrum width compared with case parents (P=0.042). In addition, mothers in the case group had an increased prevalence of narrow philtrum (P=0.001), the cleft risk was associated with a reduced odds ratio if the mother had an average philtrum width (OR 0.65, P=0.01). The results of the effect of NSCL/P SNPs on normal variation amongst the ALSPAC cohort (Chapter 7) also demonstrated that a narrow philtrum width was associated with the combined NSCL/P genetic allele score (p=0.0003), and in addition, surfaced during single SNP analysis; rs7590268 (P=0.002) and rs13041247 (P=0.019). Various facial width phenotypes have been suggested previously as a potential discriminator between case and control patients, with increased upper facial width, mouth width (Yoon, Y.J. *et al*, 2004, Weinberg, S.M. *et al*, 2008b) and philtrum width

(Defay, D.K., 2011) occurring at a higher incidence amongst unaffected mothers of cleft children.

Case parents also had increased prevalence of absent lower lip border (P=0.008) and double border (P=0.001). The ALSPAC cohort (Chapter 7) demonstrated that SNPs rs6478391 (*FOXE1*) and rs7632427 (*EPHA3*) were associated with lack of vermilion border (Figure 7.3). In addition, rs4752028 (*VAX1*) was associated with lack of double vermilion border (P=0.04).

The most significant association that was observed amongst the ALSPAC cohort (Chapter 7) was with skeletal pattern and rs227731 (*NOG*) ($P=1.58x10^{-6}$), which induced a skeletal II pattern. Conversely, in this study, case parents were more likely to have skeletal I patterns (P=0.03). Skeletal variations have been associated with a number of previous studies, with some suggesting an increased prevalence of skeletal II patterns (Prochazkova, J. *et al*, 1986), whilst others reporting increased prevalence of skeletal III (Mossey, P.A. *et al*, 1997).

8.4.2 Sex specific phenotypes associated with increased prevalence amongst cleft parents

Several authors have suggested different risk features amongst mothers and fathers (Yoon, Y.J. *et al*, 2004, Weinberg, S.M. *et al*, 2009).

In this study, there were nine phenotypes that showed sex predilection amongst case parents, and the majority of these were associated with phenotypes of the mother.

Mothers of affected offspring had increased prevalence of narrow philtrum (P=0.012), convex upper lip contours (P=0.03), upper lip groove (P=0.05), absent lower lip groove (P=0.004), flat lower lip contour (P=0.009) and angular lower lip tone with bumping (P=0.002) (Figure 8.2 and Table 8.3). In previous research, mothers have demonstrated increased upper facial width (Weinberg, S.M. *et al*, 2009).

Fathers on the other hand, had increased prevalence of absent lower lip border (P=0.015) and lower lip double borders (P=0.003) (Figure 8.2 and Table 8.3). In previous research, fathers have demonstrated increased cranial base width, increased lower facial height and decreased upper facial height compared with controls (Weinberg, S.M. *et al*, 2009). Proposing that differences characterising unaffected relatives are partly sex-specific (Weinberg, S.M. *et al*, 2009).

8.5 Conclusion

The objectives were to:

- Perform a case/control study to assess the prevalence of lip phenotypes amongst unaffected biological parents of cleft and non-cleft children
- Assess the predictive capability of lip phenotypes as a precursor of cleft risk

Certain lip phenotypes have a higher incidence amongst parents with NSCL/P offspring, specifically, narrow philtrum width (mothers), thin upper lips (fathers), convex upper lip contour, upper lip groove (mothers) and flat or narrow at midline lower lip contours.

Logistic regression revealed increased odds ratios with six phenotypes amongst mothers and three phenotypes amongst fathers. Amongst case mothers, phenotypes included a narrow philtrum (OR: 1.66, P=0.024); convex upper lip contours (OR: 2.64, P=0.007); upper lip groove (OR: 2.36, P=0.034); absent lower lip groove (OR: 2.85, P=0.002); flat lower lip contour (OR: 2.15, P=0.007) and lower lip tone with bilateral bumping (OR: 2.55, P=0.001). Whilst fathers on the other hand, had increased odds rations associated with thin upper lips (P=0.03), absent lower lip vermilion border (OR: 2.42, P=0.006) and absent lower lip double border (OR: 2.25, P=0.002). This study further supports the notion that phenotype differences characterising unaffected relatives are partly sex-specific (Weinberg, S.M. *et al*, 2009).

Narrow philtrum, upper lip groove, absent vermilion borders and absent upper lip drop are all features that were also observed with increased prevalence of NSCL/P SNPs in the ALSPAC data.

The results of this study further suggest that these features may be considered as markers of NSCL/P risk. In addition, there is variation in the frequency of phenotypes amongst males and females, which may suggest that a certain combination of phenotypes predisposes one to NSCL/P.

One of the main limitations of this study is that it is not known whether one case parent was influencing the genetic susceptibility, or whether both parents were contributing. Thus, in order to confirm the susceptibility phenotype, it would be necessary to genotype the parents to check the number of susceptibility cleft loci, and confirm which one was contributing towards the genetic risk.

Chapter 9: Overall Summary

The aim of this study was to investigate the biological basis of lip phenotypes.

The objectives were to:

- Develop a robust, reproducible classification system of lip phenotypes
- Measure the prevalence of lip phenotypes within a population sample of 4,747
- Assess for associations of lip phenotypes with other lip phenotypes
- Assess if there were any sex variations of lip phenotypes
- Assess the reproducibility of the classification system with other acquisition methods
- Perform a genome-wide association study (GWAS) of lip phenotypes
- Assess for associations of non-syndromic cleft lip/palate (NSCL/P) single nucleotide polymorphs (SNPs) to lip phenotypes
- Perform a case/control study to assess the prevalence of lip phenotypes amongst unaffected biological parents of cleft and non-cleft children
- Assess the predictive capability of lip phenotypes as a precursor of cleft risk

The overall aim of this study was to investigate the biological basis of lip phenotypes, in order to achieve this, a number of objectives were set.

It was initially identified that little literature existed, which could fully characterise lip phenotypes that existed in the population. In order to overcome this, a robust, reliable lip categorisation scale was developed, which had good intra and inter-examiner reliability. The study has also demonstrated that there is considerable variation in normal lip morphology. Some of these features are not amenable to classification by landmarking or measurement, but by identifying and categorising according to anatomical shapes and surface topography.

Following the development of the scale, 4,747 3D laser facial scans of individuals from the ALSPAC study were assessed and categorised according to the scale. The prevalence of the various lip phenotypes was documented (Table 4.4), and this demonstrated that there was considerable lip variation in a population of 15 year olds. Some rare phenotypes have been reported that have not been described previously. The epidemiological study is comparable to similar studies for philtrum shape, lip thickness, chin contour and skeletal pattern. The variation of the philtrum shape is greater than reported previously (Mori, A. *et al*, 2005). Skeletal classifications in this study are based on a judgement of the relative position of the upper lip to the chin in which the 3D facial shells were registered. The majority of previous studies assessing

the prevalence of skeletal patterns have been based on measurements taken from lateral cephalograms.

Previous research has postulated that some lip phenotypes are associated with other phenotypes (Carey, J.C. *et al*, 2009), though these were based on expert opinion (Table 1.5). This study confirmed that the majority of lip phenotypes are associated with other lip phenotypes (Table 4.5), with the exception of mentolabial fold and chin dimple, which appear to only be associated with skeletal pattern and lower lip tone.

The study also confirmed that there is some sex dimorphism of lip phenotypes. Males generally have angular lower lip phenotypes and wide philtrum, whilst females have narrow philtrum and defined Cupid's bow shapes. Five features were independent of sex: upper lip groove/drop, lower lip border, fullness, and mentolabial fold. Morphological associations between sexes also vary (Table 4.6), with females having strong associations with features of the philtrum and upper lip. Males have more association with lower lip contour and Cupid's bow compared with females.

As part of this study, the robustness of the scale was also tested for its use for images captured using 3D laser and 3dMD, and for greyscale and colour texture images. The reason this was carried out was to ensure that there was a means of transferring the scale to other media, in order to increase the sample size, which is necessary for replication in GWAS studies. The scale shows a high level of reliability with most of the features using 3D laser grey images (Tables 4.3 and 5.2), however, the study demonstrated that some of the surface detail is lost with 3dMD images, and an adapted scale must be employed when using colour texture images to compensate.

Once a novel means of assessing lip phenotypes had been developed, Genome-wide association analysis was performed, in an attempt to match phenotype to genotype. Genome-wide significance was reached with two phenotype/genotype associations and there were 29 close-hit associations with 18 lip phenotypes (Table 6.3). *DOCK1* was found to be associated with the phenotype "chin dimple" ($P=2x10^{-8}$) and *CDH4* was associated with a mentolabial fold ($P=1x10^{-8}$). These two phenotypes behave in an almost entirely independent manner to other traits of the lip region (Table 4.5).

SNP rs11017876 (*CDH4*) was associated with a mentolabial fold ($P=1x10^{-8}$). *CDH4* is part of the cadherin family, which has been previously associated with lip development (Du, C. *et al*, 2011). It may also have a role in brain segmentation and neuronal outgrowth, as well as muscle development. Cadherin-4 has also been observed in the

myoblastic cells of the mandibular arch, during early embryological development in chick embryos (Mootoosamy, R.C. *et al*, 2002, Nogueira, J.M. *et al*, 2015).

Replication of the two GWAS significant associations was attempted using digital photographs of an independent sample, however, it was not achieved (Table 6.4).

This study also identified an association with *ZFR* and chin dimple ($8x10^{-7}$), *ZFR* is a member of the zinc finger binding protein family, which are a diverse group of proteins that serve as mediators, binding DNA and RNA proteins. In a sample of individuals using a self-reporting method (Eriksson, N. *et al*, 2012), chin dimple was associated with *ZEB2*, which is also a zinc finger protein.

There were nine other potentially interesting associations with near cleft susceptibility regions (Table 6.5). The SNP rs10463015 arising in 5q35.1 was found to be associated with lower lip vermilion border ($P=5.3x10^{-7}$) in this ALSPAC sample. This locus has also been associated with normal facial variations (nasion) in a population of 5,388 Caucasians (Liu, F. *et al*, 2012c).

The locus 8q24 has been robustly associated with cleft susceptibility in a number of studies; it has also been associated with normal facial variation (bizygomatic distance P=0.017) (Boehringer, S. *et al*, 2011). In this sample, it was associated with lower lip tone ($P=3.3x10^{-7}$). An association was also observed between lower lip double vermilion border and the gene *RUVBL1* ($P=2.4x10^{-7}$), which is known to interact with gene *MYC*, which is also located at 8q24 (Si, J. *et al*, 2010).

Given the fact that nine regions of the lips were associated with cleft susceptibility loci, and that many authors have suggested that NSCL/P SNPs occur as common variants amongst the general population. It seemed reasonable to perform a candidate SNP association study, in which 17 previously GWAS associated NSCL/P SNPs were tested for association with lip phenotypes.

Candidate SNP associations were observed with several lip phenotypes (Table 7.4). A narrow philtrum, V-shaped Cupid's bow, absent upper lip drop, absent upper lip border, presence of an upper lip double border, thin lower lip, absence of lower lip vermilion border, absent lower lip drop and absent lower lip double border, skeletal II pattern, marked angular lower lip-chin shape and marked lower lip tone with bumping were all observed.

A NSCL/P genetic allele score was generated, which indicated that a V-shaped Cupid's bow (P=0.0002) and a narrow philtrum width (P=0.0003) were associated with multiple NSCL/P genetic alleles

In addition, two variants were suggestive of having a role to play in determining a narrow philtrum: *THADA* (P=0.002) and *MAFB* (P=0.019). *MAFB* has previously been shown to have a link with mouth width (Miller, S.F. *et al*, 2014a).

This most significant association was observed between skeletal II pattern (mandibular retrognathia) and SNP rs227731 ($P=1.58x10^{-6}$), which is likely to affect the *NOG* gene (Mangold, E. *et al*, 2010, Ludwig, K.U. *et al*, 2012). Previous research has suggested that *NOG* may play a role in mandibular development (Matsui, M. *et al*, 2014), and that parents of children with CP were more likely to have mandibular retrusion (Prochazkova, J. *et al*, 1986).

NOG encodes the noggin protein, which is an antagonist of members of the transforming growth factor beta (*TGF-ß*) superfamily, which includes bone morphogenetic protein 4 (*BMP4*). *BMP4* has been shown to regulate palatogenesis (Zhang, T.X. *et al*, 2012), and they play an important role during lip development (Chapter 2 and Table 2.1).

MAFB has been previously associated with the position of the chin (Miller, S.F. *et al*, 2014b), and this study also established associations between *MAFB* and three features of the lower lip; flat lower lip contour, full border and presence of a lower lip drop.

Given that cleft risk loci arise in the normal population, and this study highlighted possible associations between these and normal lip phenotypes, it seems plausible that unaffected family members of cleft individuals may carry a higher number of these lip phenotypes. In fact, many authors have previously postulated that unaffected parents or siblings of cleft individuals have altered facial phenotypes (Mossey, P.A. *et al*, 1997, Yoon, Y.J. *et al*, 2004, Neiswanger, K. *et al*, 2007, Weinberg, S.M. *et al*, 2008b, Weinberg, S.M. *et al*, 2009, Boehringer, S. *et al*, 2011{Defay, 2011 #632);Miller, 2014 #408}(Leslie, E.J. *et al*, 2016). A study was performed on 597 images of case-parent trios (286) and (311) controls captured using 3dMD from the FaceBase consortium. Nine phenotypes were associated with an increased prevalence amongst unaffected parents of cleft offspring. There was a reduced prevalence of average philtrum width amongst unaffected parents of cleft children (65% controls, 54% case, (P=0.042)). Case mothers had a higher prevalence of narrow philtrum (38%) compared with (27%)

controls (P=0.001). Previous research has identified an increased upper facial width; including an increased mouth width amongst unaffected relatives (Yoon, Y.J. *et al*, 2004, Weinberg, S.M. *et al*, 2008b).

This study also identified that unaffected mothers of cleft children had increased prevalence of up to six lip phenotypes encompassing regions of the philtrum, upper lip, lower lip and sub-lip, whilst unaffected fathers had increased prevalence of up to three phenotypes, involving only the lower lip and sub-lip region. This supports findings from previous research, which demonstrated that the phenotypes characterising unaffected relatives are partly sex-specific (Weinberg, S.M. *et al*, 2009).

The genotype analysis performed during this thesis identified that out of 25 lip phenotypes, 21 were associated with NSCLP SNPs. The case/control study demonstrated that only 9 out of 23 lip phenotypes were associated with unaffected parents of cleft children. Analysis of the lip phenotypes that arose in both studies identified a consistent relationship of cleft risk for 7 lip phenotypes: narrow philtrum, upper lip groove, absent upper lip drop, absent upper lip vermilion border, absent lower lip vermilion border, lower lip double border and lower lip tone with bumping (Table 7.4 and Table 8.3).

This study has demonstrated that NSCL/P SNPs appear to affect lip phenotypes, and in addition, unaffected parents of cleft children, who one could assume would be carrying NSCLP NSCL/P genetic alleles, also have a higher prevalence of certain lip phenotypes.

Therefore, the lip phenotypes - narrow philtrum, upper lip groove, absent upper lip drop, absent upper lip vermilion border, absent lower lip vermilion border, lower lip double border and lower lip tone with bumping could be considered as markers of subclinical NSCL/P phenotypes.

Conclusions

The biological basis of lip phenotypes has been investigated, resulting in the following conclusions:

- The lip categorisation scale developed as part of this study was demonstrated to be a robust tool, with good intra and inter-examiner reliability.
- Considerable lip variation exists in a population of 15 year olds, with some rare phenotypes not previously identified.
- There is sex dimorphism, with males generally having more angular lower lip phenotypes and wide philtrum, whilst females have less angular phenotypes, with a narrow philtrum and defined Cupid's bow shapes. Five features were independent of sex: upper lip groove/drop, lower lip border, fullness, and mentolabial fold.
- The lip categorisation scale, developed as part of this study, was capable of modification, in order to assess lip phenotypes using different acquisition techniques (3dMD) and surface texture (colour/grey).
- The robustness of the lip scale and significant prevalence of lip phenotypes provided a good foundation to explore phenotype/genotype associations in genome-wide association studies (GWAS).
- Statistically significant associations of *DOCK1* with chin dimple ($P=2x10^{-8}$), and *CDH4* with mentolabial fold ($P=1x10^{-8}$) were discovered.
- Validity of GWAS discovery associations, with nine located in cleft susceptibility loci.
- NSCL/P SNPs appear to affect lip phenotypes, causing a narrow philtrum, V-shaped Cupid's bow and skeletal II pattern.
- A statistically significant association of *NOG* with skeletal II pattern was discovered ($P=1.58x10^{-6}$).
- Parents of cleft children have different lip phenotypes to those of controls, having a higher prevalence of narrow philtrum, upper lip groove and absent vermilion borders.

Chapter 10: Future Studies

This study has demonstrated that there is considerable variation in lip phenotypes, which is not amenable to classification by landmarking or other methods. These phenotypes are likely to arise due to many genetic variations.

There are many research questions that have arisen as a result of this work, and the potential for future research.

10.1 Validate discovery GWAS

A limiting factor of this study was a lack of availability of individuals with 3D data along with genotype accessibility, in order to replicate which genes affect lip phenotypes and further explore the near hit GWAS results. So that larger population samples can be tested, it will be necessary to assess the capability of using the classification scale (Figure 4.4) using further acquisition techniques, such as digital photographs and direct clinical assessment.

The scale has been adapted in order to enable assessment of lip phenotypes using good quality digital photographs. A dataset of 2,000 individuals from Oulu, Northern Finland has been shared with Cardiff University, and phenotyping is well underway. All of these individuals have genotyped data available, enabling an opportunity to attempt replication of the GWAS discovery findings, and therefore validate these results.

10.2 Automated lip phenotype recognition

Most of the lip phenotypes identified in this study, have been recorded according to surface texture (philtrum shape, vermilion border, mentolabial fold, chin dimple etc.). It is conceivable that a computer programme could be developed to automatically register these lip phenotypes, thereby removing human error, and increasing the statistical reliability.

A computer science PhD student at Cardiff University (Hawraa Abbas) has developed a computer software programme, which automatically clusters individuals according to variations in the phenotypes. These have significant crossover with the phenotypes described in the classification system (Figure 4.4 and Table 4.2). This method of categorising the ALSPAC individuals is a means of validating the classification system

employed in this study, and will also remove any examiner error, improving the accuracy of cataloguing the phenotypes.

In addition to this, many of the phenotypes appear to be associated with other seemingly unrelated phenotypes, suggestive of pleotropy. This methodology could be further expanded to include the whole face, and categorise individuals according to commonly occurring facial shapes.

10.3 Age-related changes

There have been many studies that have recorded age-related changes in terms of height and thickness of the lip (Nanda, R.S. *et al*, 1990, Ferrario, V.F. *et al*, 2000, Bergman, R.T. *et al*, 2014), however little is known as to how characteristic features alter with age.

A Welsh and Finnish cohort of schoolchildren, aged between 11-14yrs was available for assessment of age-related changes. The results of this study are due to be submitted for publication.

10.4 Facial changes following orthognathic surgery

Orthognathic surgery is a term used to describe corrective jaw surgery, in which the patient presents to the Orthodontist with concerns relating to function (speech or masticatory problems) and/or aesthetics. Treatment involves a period of orthodontic care alignment initially to position the teeth in the correct position within the jaw, followed by a surgical procedure to correct the jaw alignment. During orthodontic planning, a decision is made as to where the teeth should be positioned in order to provide the ideal aesthetic facial result, which is also stable and functional (Tompach, P.C. *et al*, 1995). Unfavorable changes in lip thickness may occur as a result of surgery (Abeltins, A. *et al*, 2011), however, calculating the reaction of the soft tissues following this procedure is currently unpredictable (Ahmad Akhoundi, M.S. *et al*, 2012, Wermker, K. *et al*, 2014).

Future research would involve recruiting orthognathic patients, and assessing their lip phenotypes prior to, and following surgery, in order to assess how different lip phenotypes react to surgery.

An additional research question in this area would be to assess whether any of these lip phenotypes contribute towards the risk of relapse.

10.5 FAS

FAS can be challenging to diagnose, due to its varying clinical and behavioural presentation (Astley, S.J. *et al*, 1999, Morleo, M. *et al*, 2011). It would be useful to conduct a further study to assess the effect that maternal alcohol consumption has on lip phenotypes.

The ALSPAC dataset holds information about the mother's alcohol consumption during pregnancy, and ethical approval for this study has been approved.

10.6 Different ethnicities

Many studies have suggested variations in lip dimensions amongst individuals of different ethnic groups (Lew, K.K. *et al*, 1993, Wilkinson, C.M. *et al*, 2003, Zhu, L.Y. *et al*, 2008, Seager, D.C. *et al*, 2009, Wong, W.W. *et al*, 2010). The classification scale could be used to assess the prevalence of features amongst different ethnic groups, and to assess whether there are any other unique features present, which were not captured using this population sample.

Following phenotyping these different population groups, it was also be possible to perform GWAS on them, in order to assess whether there is any genetic crossover between different populations. In addition, this may highlight why different ethnic groups have a higher incidence of cleft compared to other populations.

10.7 Subclinical cleft markers

In addition, NSCL/P SNPs appear to affect lip phenotypes, and unaffected parents of cleft children have increased prevalence of some phenotypes.

One of the main limitations of this study is that it is not known whether one parent was influencing the genetic susceptibility, or whether both parents were contributing.

Thus, in order to confirm the susceptibility phenotype, it would be necessary to genotype the parents to check the number of susceptibility cleft loci, and confirm which one was contributing towards the genetic risk.

With further research, it may be possible to identify individuals or couples, who have a higher risk of having cleft offspring, facilitating the development of a robust, reliable classification system of subclinical cleft markers.

Bibliography

ABEL, E.L. 1995. An update on incidence of FAS: FAS is not an equal opportunity birth defect. *Neurotoxicology and Teratology,* 17**,** 437-443.

ABELTINS, A. & JAKOBSONE, G. 2011. Soft tissue thickness changes after correcting Class III malocclusion with bimaxillar surgery. *Stomatologija,* 13**,** 87-91.

AHLGREN, S.C. & BRONNER-FRASER, M. 1999. Inhibition of sonic hedgehog signaling in vivo results in craniofacial neural crest cell death. *Current Biology,* 9**,** 1304-1314.

AHLGREN, S.C. THAKUR, V. & BRONNER-FRASER, M. 2002. Sonic hedgehog rescues cranial neural crest from cell death induced by ethanol exposure. *Proceedings of the National Academy of Sciences,* 99**,** 10476-10481.

AHMAD AKHOUNDI, M.S. SHIRANI, G. ARSHAD, M. HEIDAR, H. & SODAGAR, A. 2012. Comparison of an Imaging Software and Manual Prediction of Soft Tissue Changes after Orthognathic Surgery. *Journal of Dentistry,* 9**,** 178-187.

AHUJA, R. PINYOL, R. REICHENBACH, N. CUSTER, L. KLINGENSMITH, J. KESSELS, M.M. & QUALMANN, B. 2007. Cordon-bleu is an actin nucleation factor and controls neuronal morphology. *Cell,* 131**,** 337-350.

AL ALI, A. RICHMOND, S. POPAT, H. TOMA, A.M. PLAYLE, R. ZHUROV, A.I. MARSHALL, D. ROSIN, P.L. & HENDERSON, J. 2014. The influence of asthma on face shape: a three-dimensional study. *The European Journal of Orthodontics,* 36**,** 373-380.

ALKURAYA, F.S. SAADI, I. LUND, J.J. TURBE-DOAN, A. MORTON, C.C. & MAAS, R.L. 2006. SUMO1 haploinsufficiency leads to cleft lip and palate. *Science,* 313**,** 1751.

ALLEN, P.B. GREENFIELD, A.T. SVENNINGSSON, P. HASPESLAGH, D.C. & GREENGARD, P. 2004. Phactrs 1-4: A family of protein phosphatase 1 and actin regulatory proteins. *Proceedings of the National Academy of Sciences,* 101**,** 7187-7192.

ALLORI, A.C. MULLIKEN, J.B. MEARA, J.G. SHUSTERMAN, S. & MARCUS, J.R. 2017. Classification of Cleft Lip/Palate: Then and Now. *The Cleft Palate Craniofacial Journal* 54**,** 175-188.

ANDELFINGER, G. TAPPER, A.R. WELCH, R.C. VANOYE, C.G. GEORGE, A.L., JR. & BENSON, D.W. 2002. KCNJ2 mutation results in Andersen syndrome with sex-specific cardiac and skeletal muscle phenotypes. *The American Journal of Human Genetics,* 71**,** 663-668.

ARBOUR, L. REZAZADEH, S. ELDSTROM, J. WEGET-SIMMS, G. RUPPS, R. DYER, Z. TIBBITS, G. ACCILI, E. CASEY, B. KMETIC, A. SANATANI, S. & FEDIDA, D. 2008. A KCNQ1 V205M missense mutation causes a high rate of long QT syndrome in a First Nations community of northern British Columbia: a community-based approach to understanding the impact. *Genetics in Medicine,* 10**,** 545-550.

ARNETT, G.W. JELIC, J.S. KIM, J. CUMMINGS, D.R. BERESS, A. WORLEY, C.M. CHUNG, B. & BERGMAN, R. 1999. Soft tissue cephalometric analysis: Diagnosis and treatment planning of dentofacial deformity. *American Journal of Orthodontics and Dentofacial Orthopedics* 116**,** 239-253.

ASHIQUE, A.M. FU, K. & RICHMAN, J.M. 2002. Endogenous bone morphogenetic proteins regulate outgrowth and epithelial survival during avian lip fusion. *Development,* 129**,** 4647-4660.

ASTLEY, S.J. & CLARREN, S.K. 1995. A Fetal Alcohol Syndrome Screening Tool. *Alcoholism: Clinical and Experimental Research,* 19**,** 1565-1571.

ASTLEY, S.J. & CLARREN, S.K. 1996. A case definition and photographic screening tool for the facial phenotype of fetal alcohol syndrome. *The Journal of Pediatrics,* 129**,** 33-41.

ASTLEY, S.J. & CLARREN, S.K. 1999. Diagnostic Guide for Fetal Alcohol Syndrome and Related Conditions. *In:* NETWORK, F.D.A.P. (ed.) 2nd ed.: University of Washington.

ATTANASIO, C. NORD, A.S. ZHU, Y. BLOW, M.J. LI, Z. LIBERTON, D.K. MORRISON, H. PLAJZER-FRICK, I. HOLT, A. HOSSEINI, R. PHOUANENAVONG, S. AKIYAMA, J.A. SHOUKRY, M. AFZAL, V. RUBIN, E.M. FITZPATRICK, D.R. REN, B. HALLGRÍMSSON, B. PENNACCHIO, L.A. & VISEL, A. 2013. Fine Tuning of Craniofacial Morphology by Distant-Acting. *Science,* 342**,** 1241006.

AXELSSON, H. LONNROTH, C. WANG, W. SVANBERG, E. & LUNDHOLM, K. 2005. Cyclooxygenase inhibition in early onset of tumor growth and related angiogenesis evaluated in EP1 and EP3 knockout tumor-bearing mice. *Angiogenesis,* 8**,** 339-348.

BACCETTI, T. REYES, B.C. & JR, J.A.M. 2005. Gender Differences in Class III Malocclusion. *The Angle Orthodontist,* 75**,** 510-520.

BACHLER, M. & NEUBUSER, A. 2001. Expression of members of the Fgf family and their receptors during midfacial development. *Mechanisms of Development,* 100**,** 313-316.

BARLOW, A.J. & FRANCIS-WEST, P.H. 1997. Ectopic application of recombinant BMP-2 and BMP-4 can change patterning of developing chick facial primordia. *Development,* 124**,** 391-398.

BEATY, T.H. MARAZITA, M.L. & LESLIE, E.J. 2016. Genetic factors influencing risk to orofacial clefts: today's challenges and tomorrow's opportunities. *F1000Res,* 5.

BEATY, T.H. MURRAY, J.C. MARAZITA, M.L. MUNGER, R.G. RUCZINSKI, I. HETMANSKI, J.B. LIANG, K.Y. WU, T. MURRAY, T. FALLIN, M.D. REDETT, R.A. RAYMOND, G. SCHWENDER, H. JIN, S.C. COOPER, M.E. DUNNWALD, M. MANSILLA, M.A. LESLIE, E. BULLARD, S. LIDRAL, A.C. MORENO, L.M. MENEZES, R. VIEIRA, A.R. PETRIN, A. WILCOX, A.J. LIE, R.T. JABS, E.W. WU-CHOU, Y.H. CHEN, P.K. WANG, H. YE, X. HUANG, S. YEOW, V. CHONG, S.S. JEE, S.H. SHI, B. CHRISTENSEN, K. MELBYE, M. DOHENY, K.F. PUGH, E.W. LING, H. CASTILLA, E.E. CZEIZEL, A.E. MA, L. FIELD, L.L. BRODY, L. PANGILINAN, F. MILLS, J.L. MOLLOY, A.M. KIRKE, P.N. SCOTT, J.M. ARCOS-BURGOS, M. & SCOTT, A.F. 2010. A genome-wide association study of cleft lip with and without cleft palate identifies risk variants near MAFB and ABCA4. *Nature Genetics,* 42**,** 525-529.

BEATY, T.H. TAUB, M.A. SCOTT, A.F. MURRAY, J.C. MARAZITA, M.L. SCHWENDER, H. PARKER, M.M. HETMANSKI, J.B. BALAKRISHNAN, P. MANSILLA, M.A. MANGOLD, E. LUDWIG, K.U. NOETHEN, M.M. RUBINI, M. ELCIOGLU, N. & RUCZINSKI, I. 2013. Confirming genes influencing risk to cleft lip with/without cleft palate in a case-parent trio study. *Human Genetics,* 132**,** 771-781.

BERGMAN, R.T. WASCHAK, J. BORZABADI-FARAHANI, A. & MURPHY, N.C. 2014. Longitudinal study of cephalometric soft tissue profile traits between the ages of 6 and 18 years. *The Angle Orthodontist,* 84**,** 48-55.

BIRNBAUM, S. LUDWIG, K.U. REUTTER, H. HERMS, S. STEFFENS, M. RUBINI, M. BALUARDO, C. FERRIAN, M. ALMEIDA DE ASSIS, N. ALBLAS, M.A. BARTH, S. FREUDENBERG, J. LAUSTER, C. SCHMIDT, G. SCHEER, M. BRAUMANN, B. BERGE, S.J. REICH, R.H. SCHIEFKE, F. HEMPRICH, A. POTZSCH, S. STEEGERS-THEUNISSEN, R.P. POTZSCH, B. MOEBUS, S. HORSTHEMKE, B. KRAMER, F.J.

WIENKER, T.F. MOSSEY, P.A. PROPPING, P. CICHON, S. HOFFMANN, P. KNAPP, M. NOTHEN, M.M. & MANGOLD, E. 2009. Key susceptibility locus for nonsyndromic cleft lip with or without cleft palate on chromosome 8q24. *Nature Genetics,* 41**,** 473-477.

BISHARA, S.E. JAKOBSEN, J.R. HESSION, T.J. & TREDER, J.E. 1998. Soft tissue profile changes from 5 to 45 years of age. *American Journal of Orthodontics and Dentofacial Orthopedics,* 114**,** 698-706.

BITTNER, C. & PANCHERZ, H. 1990. Facial morphology and malocclusions. *American Journal of Orthodontics and Dentofacial Orthopedics,* 97**,** 308-315.

BLAKE, J.A. & ZIMAN, M.R. 2014. Pax genes: regulators of lineage specification and progenitor cell maintenance. *Development,* 141**,** 737-751.

BOEHRINGER, S. VAN DER LIJN, F. LIU, F. GUNTHER, M. SINIGEROVA, S. NOWAK, S. LUDWIG, K.U. HERBERZ, R. KLEIN, S. HOFMAN, A. UITTERLINDEN, A.G. NIESSEN, W.J. BRETELER, M.M. VAN DER LUGT, A. WURTZ, R.P. NOTHEN, M.M. HORSTHEMKE, B. WIECZOREK, D. MANGOLD, E. & KAYSER, M. 2011. Genetic determination of human facial morphology: links between cleft-lips and normal variation. *European Journal of Human Genetics,* 19**,** 1192-1197.

BOOKSTEIN, F.L. 1997. Landmark methods for forms without landmarks: morphometrics of group differences in outline shape. *Medical Image Analysis,* 1**,** 225-243.

BOOKSTEIN, F.L. 2001. Morphometrics. Chichester: John Wiley & Sons, Ltd.

BOTTO, L.D. ERICKSON, J.D. MULINARE, J. LYNBERG, M.C. & LIU, Y. 2002. Maternal fever, multivitamin use, and selected birth defects: evidence of interaction? *Epidemiology,* 13**,** 485-488.

BOYD, A. GOLDING, J. MACLEOD, J. LAWLOR, D.A. FRASER, A. HENDERSON, J. MOLLOY, L. NESS, A. RING, S. & DAVEY SMITH, G. 2013. Cohort Profile: the 'children of the 90s'--the index offspring of the Avon Longitudinal Study of Parents and Children. *International Journal of Epidemiology,* 42**,** 111-127.

BRAYBROOK, C. DOUDNEY, K. MARCANO, A.C. ARNASON, A. BJORNSSON, A. PATTON, M.A. GOODFELLOW, P.J. MOORE, G.E. & STANIER, P. 2001. The T-box transcription factor gene TBX22 is mutated in X-linked cleft palate and ankyloglossia. *Nature Genetics,* 29**,** 179-183.

BREWER, C.M. LEEK, J.P. GREEN, A.J. HOLLOWAY, S. BONTHRON, D.T. MARKHAM, A.F. & FITZPATRICK, D.R. 1999. A locus for isolated cleft palate, located on human chromosome 2q32. *The American Journal of Human Genetics,* 65**,** 387-396.

BRITANOVA, O. DEPEW, M.J. SCHWARK, M. THOMAS, B.L. MILETICH, I. SHARPE, P. & TARABYKIN, V. 2006. Satb2 haploinsufficiency phenocopies 2q32-q33 deletions, whereas loss suggests a fundamental role in the coordination of jaw development. *The American Journal of Human Genetics,* 79**,** 668-678.

BROADBENT, B.H. 1981. A new X-ray technique and its application to orthodontia. *The Angle Orthodontist,* 51**,** 93-114.

BRUNNER, H.G. VAN BEERSUM, S.E. WARMAN, M.L. OLSEN, B.R. ROPERS, H.H. & MARIMAN, E.C. 1994. A Stickler syndrome gene is linked to chromosome 6 near the COL11A2 gene. *Human Molecular Genetics,* 3**,** 1561-1564.

BURSTONE, C.J. 1967. Lip posture and its significance in treatment planning. *American Journal of Orthodontics,* 53**,** 262-284.

BUSH, W.S. & MOORE, J.H. 2012. Chapter 11: Genome-wide association studies. *PLoS Computational Biology,* 8**,** e1002822.

BUTLER, M.G. USREY, K.M. ROBERTS, J.L. MANZARDO, A.M. & SCHROEDER, S.R. 2013. 20q13.2-q13.33 deletion syndrome: A case report. *Journal of Pediatric Genetics,* 2**,** 157-161.

CALDWELL, T. 2014. Available: http://www.bodywisea2.com.

CAREY, J.C. COHEN, M.M. CURRY, C.J.R. DEVRIENDT, K. HOLMES, L.M. & VERLOES, A. 2009. Elements of Morphology: Standard Terminology for the Lips, Mouth, and Oral Region. *American Journal of Medical Genetics Part A,* 149A**,** 77-92.

CARINCI, F. SCAPOLI, L. PALMIERI, A. ZOLLINO, I. & PEZZETTI, F. 2007. Human genetic factors in nonsyndromic cleft lip and palate: an update. *International Journal of Pediatric Otorhinolaryngology,* 71**,** 1509-1519.

CARRE, A. HAMZA, R.T. KARIYAWASAM, D. GUILLOT, L. TEISSIER, R. TRON, E. CASTANET, M. DUPUY, C. EL KHOLY, M. & POLAK, M. 2014. A novel FOXE1 mutation (R73S) in Bamforth-Lazarus syndrome causing increased thyroidal gene expression. *Thyroid,* 24**,** 649-654.

CARROLL, E.A. GERRELLI, D. GASCA, S. BERG, E. BEIER, D.R. COPP, A.J. & KLINGENSMITH, J. 2003. Cordon-bleu is a conserved gene involved in neural tube formation. *Developmental Biology,* 262**,** 16-31.

CARROLL, T.J. PARK, J.S. HAYASHI, S. MAJUMDAR, A. & MCMAHON, A.P. 2005. Wnt9b plays a central role in the regulation of mesenchymal to epithelial transitions underlying organogenesis of the mammalian urogenital system. *Developmental Cell,* 9**,** 283-292.

CASTILLA, E.E. & MARTINEZ-FRIAS, M.L. 1995. Congenital healed cleft lip. *American Journal of Medical Genetics Part A,* 58**,** 106-112.

CHARRON, F. & TESSIER-LAVIGNE, M. 2005. Novel brain wiring functions for classical morphogens: a role as graded positional cues in axon guidance. *Development,* 132**,** 2251-2262.

CHEN, X. OVERCASH, R. GREEN, T. HOFFMAN, D. ASCH, A.S. & RUIZ-ECHEVARRIA, M.J. 2011. The tumor suppressor activity of the transmembrane protein with epidermal growth factor and two follistatin motifs 2 (TMEFF2) correlates with its ability to modulate sarcosine levels. *The Journal of Biological Chemistry,* 286**,** 16091-16100.

CHENEVIX-TRENCH, G. JONES, K. GREEN, A.C. DUFFY, D.L. & MARTIN, N.G. 1992. Cleft lip with or without cleft palate: associations with transforming growth factor alpha and retinoic acid receptor loci. *The American Journal of Human Genetics,* 51**,** 1377-1385.

CHENG, C. CUI, H. ZHANG, L. JIA, Z. SONG, B. WANG, F. LI, Y. LIU, J. KONG, P. SHI, R. BI, Y. YANG, B. WANG, J. ZHAO, Z. ZHANG, Y. HU, X. YANG, J. HE, C. ZHAO, Z. WANG, J. XI, Y. XU, E. LI, G. GUO, S. CHEN, Y. YANG, X. CHEN, X. LIANG, J. GUO, J. CHENG, X. WANG, C. ZHAN, Q. & CUI, Y. 2016. Genomic analyses reveal FAM84B and the NOTCH pathway are associated with the progression of esophageal squamous cell carcinoma. *Gigascience,* 5**,** 1.

CHRISTENSEN, K. & FOGH-ANDERSEN, P. 1993. Cleft lip (+/- cleft palate) in Danish twins, 1970-1990. *American Journal of Medical Genetics Part A,* 47**,** 910-916.

CLAES, P. LIBERTON, D.K. DANIELS, K. ROSANA, K.M. QUILLEN, E.E. PEARSON, L.N. MCEVOY, B. BAUCHET, M. ZAIDI, A.A. YAO, W. TANG, H. BARSH, G.S. ABSHER, D.M. PUTS, D.A. ROCHA, J. BELEZA, S. PEREIRA, R.W. BAYNAM, G. SUETENS, P. VANDERMEULEN, D. WAGNER, J.K. BOSTER, J.S. & SHRIVER, M.D. 2014. Modeling 3D Facial Shape from DNA. *PLoS Genetics,* 10.

COBOURNE, M.T. 2004. The complex genetics of cleft lip and palate. *The European Journal of Orthodontics,* 26**,** 7-16.

CORDERO, D.R. BRUGMANN, S. CHU, Y. BAJPAI, R. JAME, M. & HELMS, J.A. 2011. Cranial neural crest cells on the move: Their roles in craniofacial development. *American Journal of Medical Genetics Part A,* 155**,** 270-279.

COX, T. 2004. Taking it to the max: the genetic and developmental mechanisms coordinating midfacial morphogenesis and dysmorphology. *Clinical Genetics* 65**,** 163–176.

DA FONTOURA, C.S. MILLER, S.F. WEHBY, G.L. AMENDT, B.A. HOLTON, N.E. SOUTHARD, T.E. ALLAREDDY, V. & MORENO URIBE, L.M. 2015. Candidate Gene Analyses of Skeletal Variation in Malocclusion. *Journal of Dental Research,* 94**,** 913-920.

DANN, J.J., 3RD FONSECA, R.J. & BELL, W.H. 1976. Soft tissue changes associated with total maxillary advancement: a preliminary study. *Journal of Oral Surgery,* 34**,** 19-23.

DE LA CHAPELLE, A. HERVA, R. KOIVISTO, M. & AULA, P. 1981. A deletion in chromosome 22 can cause DiGeorge syndrome. *Human Genetics,* 57**,** 253-256.

DEFAY, D.K. 2011. *3D facial analysis: unaffected parents of individuals with cleft lip/palate.* Master of Science, University of Iowa.

DIGILIO, M. MARINO, B. CAPOLINO, R. & DALLAPICCOLA, B. 2005. Clinical manifestations of Deletion 22q11.2 syndrome (DiGeorge/Velo-Cardio-Facial syndrome). *Images in Paediatric Cardiology,* 7**,** 23-34.

DINESHSHANKAR, J. GANAPATHI, N. YOITHAPPRABHUNATH, T.R. MAHESWARAN, T. KUMAR, M.S. & ARAVINDHAN, R. 2013. Lip prints: Role in forensic odontology. *Journal of Pharmacy & Bioallied Sciences,* 5**,** 95-97.

DING, H. WU, X. BOSTROM, H. KIM, I. WONG, N. TSOI, B. O'ROURKE, M. KOH, G.Y. SORIANO, P. BETSHOLTZ, C. HART, T.C. MARAZITA, M.L. FIELD, L.L. TAM, P.P. & NAGY, A. 2004. A specific requirement for PDGF-C in palate formation and PDGFR-alpha signaling. *Nature Genetics,* 36**,** 1111-1116.

DIXON, M.J. MARAZITA, M.L. BEATY, T.H. & MURRAY, J.C. 2011. Cleft lip and palate: synthesizing genetic and environmental influences. *Nature Reviews. Genetics,* 12**,** 167-178.

DOBREVA, G. CHAHROUR, M. DAUTZENBERG, M. CHIRIVELLA, L. KANZLER, B. FARINAS, I. KARSENTY, G. & GROSSCHEDL, R. 2006. SATB2 is a multifunctional determinant of craniofacial patterning and osteoblast differentiation. *Cell,* 125**,** 971-986.

DODE, C. LEVILLIERS, J. DUPONT, J.M. DE PAEPE, A. LE DU, N. SOUSSI-YANICOSTAS, N. COIMBRA, R.S. DELMAGHANI, S. COMPAIN-NOUAILLE, S. BAVEREL, F. PECHEUX, C. LE TESSIER, D. CRUAUD, C. DELPECH, M. SPELEMAN, F. VERMEULEN, S. AMALFITANO, A. BACHELOT, Y. BOUCHARD, P. CABROL, S. CAREL, J.C. DELEMARRE-VAN DE WAAL, H. GOULET-SALMON, B. KOTTLER, M.L. RICHARD, O. SANCHEZ-FRANCO, F. SAURA, R. YOUNG, J. PETIT, C. & HARDELIN, J.P. 2003. Loss-of-function mutations in FGFR1 cause autosomal dominant Kallmann syndrome. *Nature Genetics,* 33**,** 463-465.

DOHVOMA, C. HUTCHISON, I. & CALVERT, M. 1995. Labiomental intertrigo. An indication for orthognathic surgery. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics,* 79**,** 551-553.

DRAVET, C. JULIAN, C. LEGRAS, C. MAGAUDDA, A. GUERRINI, R. GENTON, P. SOULAYROL, S. GIRAUD, N. MESDJIAN, E. TRENTIN, G. & ET AL. 1992. Epilepsy,

antiepileptic drugs, and malformations in children of women with epilepsy: a French prospective cohort study. *Neurology,* 42**,** 75-82.

DU, C. HUANG, T. SUN, D. MO, Y. FENG, H. ZHOU, X. XIAO, X. YU, N. HOU, B. HUANG, G. ERNBERG, I. & ZHANG, Z. 2011. CDH4 as a novel putative tumor suppressor gene epigenetically silenced by promoter hypermethylation in nasopharyngeal carcinoma. *Cancer Letters,* 309**,** 54-61.

DUMONT, N.A. WANG, Y.X. VON MALTZAHN, J. PASUT, A. BENTZINGER, C.F. BRUN, C.E. & RUDNICKI, M.A. 2015. Dystrophin expression in muscle stem cells regulates their polarity and asymmetric division. *Nature Medicine,* 21**,** 1455-1463.

EL HELOU, J. BELANGER-NELSON, E. FREYBURGER, M. DORSAZ, S. CURIE, T. LA SPADA, F. GAUDREAULT, P.O. BEAUMONT, E. POULIOT, P. LESAGE, F. FRANK, M.G. FRANKEN, P. & MONGRAIN, V. 2013. Neuroligin-1 links neuronal activity to sleep-wake regulation. *Proceedings of the National Academy of Sciences,* 110**,** 9974-9979.

ELKS, C.E. PERRY, J.R. SULEM, P. CHASMAN, D.I. FRANCESCHINI, N. HE, C. LUNETTA, K.L. VISSER, J.A. BYRNE, E.M. COUSMINER, D.L. GUDBJARTSSON, D.F. ESKO, T. FEENSTRA, B. HOTTENGA, J.J. KOLLER, D.L. KUTALIK, Z. LIN, P. MANGINO, M. MARONGIU, M. MCARDLE, P.F. SMITH, A.V. STOLK, L. VAN WINGERDEN, S.H. ZHAO, J.H. ALBRECHT, E. CORRE, T. INGELSSON, E. HAYWARD, C. MAGNUSSON, P.K. SMITH, E.N. ULIVI, S. WARRINGTON, N.M. ZGAGA, L. ALAVERE, H. AMIN, N. ASPELUND, T. BANDINELLI, S. BARROSO, I. BERENSON, G.S. BERGMANN, S. BLACKBURN, H. BOERWINKLE, E. BURING, J.E. BUSONERO, F. CAMPBELL, H. CHANOCK, S.J. CHEN, W. CORNELIS, M.C. COUPER, D. COVIELLO, A.D. D'ADAMO, P. DE FAIRE, U. DE GEUS, E.J. DELOUKAS, P. DORING, A. SMITH, G.D. EASTON, D.F. EIRIKSDOTTIR, G. EMILSSON, V. ERIKSSON, J. FERRUCCI, L. FOLSOM, A.R. FOROUD, T. GARCIA, M. GASPARINI, P. GELLER, F. GIEGER, C. GUDNASON, V. HALL, P. HANKINSON, S.E. FERRELI, L. HEATH, A.C. HERNANDEZ, D.G. HOFMAN, A. HU, F.B. ILLIG, T. JARVELIN, M.R. JOHNSON, A.D. KARASIK, D. KHAW, K.T. KIEL, D.P. KILPELAINEN, T.O. KOLCIC, I. KRAFT, P. LAUNER, L.J. LAVEN, J.S. LI, S. LIU, J. LEVY, D. MARTIN, N.G. MCARDLE, W.L. MELBYE, M. MOOSER, V. MURRAY, J.C. MURRAY, S.S. NALLS, M.A. NAVARRO, P. NELIS, M. NESS, A.R. NORTHSTONE, K.et al. 2010. Thirty new loci for age at menarche identified by a meta-analysis of genome-wide association studies. *Nature Genetics,* 42**,** 1077-1085.

ERIKSSON, N. DO, C.B. TUNG, J.Y. KIEFER, A.K. HINDS, D.A. MOUNTAIN, J.L. & FRANCKE, U. 2012. Over 250 novel associations with human morphological traits. 23andMe, Mountain View, CA.

ERMAKOV, S. ROSENBAUM, M.G. MALKIN, I. & LIVSHITS, G. 2010. Family-based study of association between ENPP1 genetic variants and craniofacial morphology. *Annals of Human Biology,* 37**,** 754-766.

FANG, J. DAGENAIS, S.L. ERICKSON, R.P. ARLT, M.F. GLYNN, M.W. GORSKI, J.L. SEAVER, L.H. & GLOVER, T.W. 2000. Mutations in FOXC2 (MFH-1), a forkhead family transcription factor, are responsible for the hereditary lymphedema-distichiasis syndrome. *The American Journal of Human Genetics,* 67**,** 1382-1388.

FANG, J. UCHIUMI, T. YAGI, M. MATSUMOTO, S. AMAMOTO, R. SAITO, T. TAKAZAKI, S. KANKI, T. YAMAZA, H. NONAKA, K. & KANG, D. 2012. Protein instability and functional defects caused by mutations of dihydro-orotate dehydrogenase in Miller syndrome patients. *Bioscience Reports,* 32**,** 631-639.

FARKAS, L.G. 1994. *Anthropometry of the head and face,* New York, Raven Press.

FARKAS, L.G. KATIC, M.J. HRECZKO, T.A. DEUTSCH, C. & MUNRO, I.R. 1984. Anthropometric proportions in the upper lip-lower lip-chin area of the lower face in young white adults. *American Journal of Orthodontics,* 86**,** 52-60.

FARKAS, L.G. & POSNICK, J.C. 1992a. Growth and development of regional units in the head and face based on anthropometric measurements. *The Cleft Palate Craniofacial Journal* 29**,** 201-229.

FARKAS, L.G. POSNICK, J.C. & HRECZKO, T.M. 1992. Growth patterns of the face: a morphometric study. *The Cleft Palate Craniofacial Journal* 29**,** 308-315.

FARKAS, L.G. POSNICK, J.C. HRECZKO, T.M. & PRON, G.E. 1992b. Growth pattern of the nasolabial region: A morphometric study. *The Cleft Palate Craniofacial Journal* 29**,** 318-324.

FARRALL, M. & HOLDER, S. 1992. Familial recurrence-pattern analysis of cleft lip with or without cleft palate. *Am J Hum Genet,* 50**,** 270-277.

FERRANTE, M.I. GIORGIO, G. FEATHER, S.A. BULFONE, A. WRIGHT, V. GHIANI, M. SELICORNI, A. GAMMARO, L. SCOLARI, F. WOOLF, A.S. SYLVIE, O. BERNARD, L. MALCOLM, S. WINTER, R. BALLABIO, A. & FRANCO, B. 2001. Identification of the gene for oral-facial-digital type I syndrome. *The American Journal of Human Genetics,* 68**,** 569-576.

FERRARIO, V.F. SFORZA, C. SCHMITZ, J.H. CIUSA, V. & COLOMBO, A. 2000. Normal growth and development of the lips: a 3-dimensional study from 6 years to adulthood using a geometric model *Journal of Anatomy,* 196 415-423.

FERRARIO, V.F. SFORZA, C. SCHMITZ, J.H. & SANTORO, F. 1999. Three-dimensional facial morphometric assessment of soft tissue changes after orthognathic surgery. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics,* 88**,** 549-556.

FIRNBERG, N. & NEUBUSER, A. 2002. FGF signaling regulates expression of Tbx2, Erm, Pea3, and Pax3 in the early nasal region. *Developmental Biology,* 247**,** 237-250.

FITZPATRICK, D. & FARRALL, M. 1993. An estimation of the number of susceptibility loci for isolated cleft palate. *J Craniofac Genet Dev Biol,* 13**,** 230-235.

FITZPATRICK, D.R. CARR, I.M. MCLAREN, L. LEEK, J.P. WIGHTMAN, P. WILLIAMSON, K. GAUTIER, P. MCGILL, N. HAYWARD, C. FIRTH, H. MARKHAM, A.F. FANTES, J.A. & BONTHRON, D.T. 2003. Identification of SATB2 as the cleft palate gene on 2q32-q33. *Human Molecular Genetics,* 12**,** 2491-2501.

FOSTER, J.W. DOMINGUEZ-STEGLICH, M.A. GUIOLI, S. KWOK, C. WELLER, P.A. STEVANOVIC, M. WEISSENBACH, J. MANSOUR, S. YOUNG, I.D. GOODFELLOW, P.N. & ET AL. 1994. Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. *Nature,* 372**,** 525-530.

FOSTER, T.D. & DAY, A.J. 1974. A survey of malocclusion and the need for orthodontic treatment in a Shropshire school population. *British Journal of Orthodontics,* 1**,** 73-78.

FRANCIS-WEST, P.H. ROBSON, L. & EVANS, D.J. 2003. Craniofacial development: the tissue and molecular interactions that control development of the head. *Advances in Anatomy, Embryology, and Cell Biology,* 169**,** III-VI, 1-138.

FRANCOMANO, C.A. LIBERFARB, R.M. HIROSE, T. MAUMENEE, I.H. STREETEN, E.A. MEYERS, D.A. & PYERITZ, R.E. 1987. The Stickler syndrome: evidence for close linkage to the structural gene for type II collagen. *Genomics,* 1**,** 293-296.

FRANZ, M.L. & SOKOL, A.B. 1972. The proportioned philtrum: a helpful measurement and ratio. *The Cleft Palate Journal,* 9**,** 143-146.

FRASER, F.C. 1955. Thoughts on the etiology of clefts of the palate and lip. *Acta Genetica et Statistica Medica,* 5**,** 358-369.

FRAZIER-BOWERS, S. RINCON-RODRIGUEZ, R. ZHOU, J. ALEXANDER, K. & LANGE, E. 2009. Evidence of linkage in a Hispanic cohort with a Class III dentofacial phenotype. *Journal of Dental Research,* 88**,** 56-60.

FREBOURG, T. OLIVEIRA, C. HOCHAIN, P. KARAM, R. MANOUVRIER, S. GRAZIADIO, C. VEKEMANS, M. HARTMANN, A. BAERT-DESURMONT, S. ALEXANDRE, C. LEJEUNE DUMOULIN, S. MARRONI, C. MARTIN, C. CASTEDO, S. LOVETT, M. WINSTON, J. MACHADO, J.C. ATTIE, T. JABS, E.W. CAI, J. PELLERIN, P. TRIBOULET, J.P. SCOTTE, M. LE PESSOT, F. HEDOUIN, A. CARNEIRO, F. BLAYAU, M. & SERUCA, R. 2006. Cleft lip/palate and CDH1/E-cadherin mutations in families with hereditary diffuse gastric cancer. *Journal of Medical Genetics,* 43**,** 138-142.

GARCIA, A.M. 1998. Occupational exposure to pesticides and congenital malformations: a review of mechanisms, methods, and results. *American Journal of Industrial Medicine,* 33**,** 232-240.

GARCIA BARTELS, N. NEUMANN, L.M. MLECZKO, A. RUBACH, K. PETERS, H. ROSSI, R. STERRY, W. & BLUME-PEYTAVI, U. 2007. Hay-Wells syndrome in a child with mutation in the TP73L gene. *Journal of the German Society of Dermatology,* 5**,** 919-923.

GARCIA DE MITCHELL, C.A. PESSA, J.E. SCHAVERIEN, M.V. & ROHRICH, R.J. 2008. The philtrum: anatomical observations from a new perspective. *Plastic and Reconstructive Surgery,* 122**,** 1756-1760.

GHODDOUSI, H. EDLER, R. HAERS, P. WERTHEIM, D. & GREENHILL, D. 2007. Comparison of three methods of facial measurement. *International Journal of Oral and Maxillofacial Surgery,* 36**,** 250-258.

GILDESTAD, T. BJORGE, T. VOLLSET, S.E. KLUNGSOYR, K. NILSEN, R.M. HAALAND, O.A. & OYEN, N. 2015. Folic acid supplements and risk for oral clefts in the newborn: a population-based study. *The British Journal of Nutrition,* 114**,** 1456-1463.

GOLDING, J. PEMBREY, M. JONES, R. & ALSPAC STUDY TEAM 2001 ALSPAC--the Avon Longitudinal Study of Parents and Children. I. Study methodology. *Paediatric and Perinatal Epidemiology,* 15**,** 74-87.

GONG, S.G. & GUO, C. 2003. Bmp4 gene is expressed at the putative site of fusion in the midfacial region. *Differentiation,* 71**,** 228-236.

GORLIN RJ, C., HENNEKAM RCM 2001. *Syndromes of the head and neck,* New York, Oxford Univ

Press.

GOYAL, P. BEHRING, A. KUMAR, A. & SIESS, W. 2011. STK35L1 associates with nuclear actin and regulates cell cycle and migration of endothelial cells. *PLoS One,* 6**,** e16249.

GRANT, S.F. WANG, K. ZHANG, H. GLABERSON, W. ANNAIAH, K. KIM, C.E. BRADFIELD, J.P. GLESSNER, J.T. THOMAS, K.A. GARRIS, M. FRACKELTON, E.C. OTIENO, F.G. CHIAVACCI, R.M. NAH, H.D. KIRSCHNER, R.E. & HAKONARSON, H. 2009. A genome-wide association study identifies a locus for nonsyndromic cleft lip with or without cleft palate on 8q24. ***The Journal of Pediatrics****,* 155**,** 909-913.

GRIPP, K.W. WOTTON, D. EDWARDS, M.C. ROESSLER, E. ADES, L. MEINECKE, P. RICHIERI-COSTA, A. ZACKAI, E.H. MASSAGUE, J. MUENKE, M. & ELLEDGE, S.J. 2000. Mutations in TGIF cause holoprosencephaly and link NODAL signalling to human neural axis determination. *Nature Genetics,* 25**,** 205-208.

GROSEN, D. BILLE, C. PEDERSEN, J.K. SKYTTHE, A. MURRAY, J.C. & CHRISTENSEN, K. 2010a. Recurrence risk for offspring of twins discordant for oral cleft: a population-based cohort study of the Danish 1936-2004 cleft twin cohort. *American Journal of Medical Genetics Part A,* 152A**,** 2468-2474.

GROSEN, D. CHEVRIER, C. SKYTTHE, A. BILLE, C. MOLSTED, K. SIVERTSEN, A. MURRAY, J.C. & CHRISTENSEN, K. 2010b. A cohort study of recurrence patterns among more than 54,000 relatives of oral cleft cases in Denmark: support for the multifactorial threshold model of inheritance. *Journal of Medical Genetics,* 47**,** 162-168.

GUDAS, L.J. & WAGNER, J.A. 2011. Retinoids regulate stem cell differentiation. *Journal of Cellular Physiology,* 226**,** 322-330.

GUTIERREZ, S.J. GOMEZ, M. REY, J.A. OCHOA, M. GUTIERREZ, S.M. & PRIETO, J.C. 2010. Polymorphisms of the noggin gene and mandibular micrognathia: a first approximation. *Acta Odontologica Latinoamericana,* 23**,** 13-19.

HAJEER, M.Y. MILLETT, D.T. AYOUB, A.F. & SIEBERT, J.P. 2004. Applications of 3D imaging in orthodontics: part I. *Journal of Orthodontics,* 31**,** 62-70.

HALAZONETIS, D.J. 2007. Morphometric correlation between facial soft-tissue profile shape and skeletal pattern in children and adolescents. *American Journal of Orthodontics and Dentofacial Orthopedics,* 132**,** 450-457.

HARVILLE, E.W. WILCOX, A.J. LIE, R.T. VINDENES, H. & ABYHOLM, F. 2005. Cleft lip and palate versus cleft lip only: are they distinct defects? *American Journal of Epidemiology,* 162**,** 448-453.

HASTBACKA, J. DE LA CHAPELLE, A. MAHTANI, M.M. CLINES, G. REEVE-DALY, M.P. DALY, M. HAMILTON, B.A. KUSUMI, K. TRIVEDI, B. WEAVER, A. & ET AL. 1994. The diastrophic dysplasia gene encodes a novel sulfate transporter: positional cloning by fine-structure linkage disequilibrium mapping. *Cell,* 78**,** 1073-1087.

HEIKE, C.L. UPSON, K. STUHAUG, E. & WEINBERG, S.M. 2010. 3D digital stereophotogrammetry: a practical guide to facial image acquisition. *Head & Face Medicine,* 6**,** 18.

HENNEKAM, R.C. CORMIER-DAIRE, V. HALL, J.G. MÉHES, K. PATTON, M. & STEVENSON, R.E. 2009. Elements of morphology: Standard terminology for the nose and philtrum. *American Journal of Medical Genetics Part A,* 149A**,** 61-76.

HINRICHSEN, K. 1985. The early development of morphology and patterns of the face in the human embryo. *Advances in Anatomy, Embryology, and Cell Biology,* 98**,** 1-79.

HINTSCH, G. ZURLINDEN, A. MESKENAITE, V. STEUBLE, M. FINK-WIDMER, K. KINTER, J. & SONDEREGGER, P. 2002. The calsyntenins--a family of postsynaptic membrane proteins with distinct neuronal expression patterns. *Molecular and Cellular Neurosciences,* 21**,** 393-409.

HOLLWAY, G.E. SUTHERS, G.K. HAAN, E.A. THOMPSON, E. DAVID, D.J. GECZ, J. & MULLEY, J.C. 1997. Mutation detection in FGFR2 craniosynostosis syndromes. *Human Genetics,* 99**,** 251-255.

HONEIN, M.A. RASMUSSEN, S.A. REEFHUIS, J. ROMITTI, P.A. LAMMER, E.J. SUN, L. & CORREA, A. 2007. Maternal smoking and environmental tobacco smoke exposure and the risk of orofacial clefts. *Epidemiology,* 18**,** 226-233.

HOPFER, U. FUKAI, N. HOPFER, H. WOLF, G. JOYCE, N. LI, E. & OLSEN, B.R. 2005. Targeted disruption of Col8a1 and Col8a2 genes in mice leads to anterior segment abnormalities in the eye. *FASEB Journal,* 19**,** 1232-1244.

HOWARD, T.D. PAZNEKAS, W.A. GREEN, E.D. CHIANG, L.C. MA, N. ORTIZ DE LUNA, R.I. GARCIA DELGADO, C. GONZALEZ-RAMOS, M. KLINE, A.D. & JABS, E.W. 1997. Mutations in TWIST, a basic helix-loop-helix transcription factor, in Saethre-Chotzen syndrome. *Nature Genetics,* 15**,** 36-41.

HU D, H.J. 1999. The role of sonic hedgehog in normal and abnormal craniofacial morphogenesis. *Development,* 126**,** 4873-4884.

HWANG, K. KIM, D.J. & HWANG, S.H. 2007. Musculature of the pars marginalis of the upper orbicularis oris muscle. *The Journal of Craniofacial Surgery,* 18**,** 151-154.

INGHAM, P.W. & MCMAHON, A.P. 2001. Hedgehog signaling in animal development: paradigms and principles. *Genes & Development,* 15**,** 3059-3087.

IVAN PEREZ, S. BERNAL, V. & GONZALEZ, P.N. 2006. Differences between sliding semi-landmark methods in geometric morphometrics, with an application to human craniofacial and dental variation. *Journal of Anatomy,* 208**,** 769-784.

IWASE, S. LAN, F. BAYLISS, P. DE LA TORRE-UBIETA, L. HUARTE, M. QI, H.H. WHETSTINE, J.R. BONNI, A. ROBERTS, T.M. & SHI, Y. 2007. The X-linked mental retardation gene SMCX/JARID1C defines a family of histone H3 lysine 4 demethylases. *Cell,* 128**,** 1077-1088.

JAKOBSEN, L.P. KNUDSEN, M.A. LESPINASSE, J. GARCIA AYUSO, C. RAMOS, C. FRYNS, J.P. BUGGE, M. & TOMMERUP, N. 2006. The genetic basis of the Pierre Robin Sequence. *The Cleft Palate Craniofacial Journal* 43**,** 155-159.

JENSEN, L.R. AMENDE, M. GUROK, U. MOSER, B. GIMMEL, V. TZSCHACH, A. JANECKE, A.R. TARIVERDIAN, G. CHELLY, J. FRYNS, J.P. VAN ESCH, H. KLEEFSTRA, T. HAMEL, B. MORAINE, C. GECZ, J. TURNER, G. REINHARDT, R. KALSCHEUER, V.M. ROPERS, H.H. & LENZNER, S. 2005. Mutations in the JARID1C gene, which is involved in transcriptional regulation and chromatin remodeling, cause X-linked mental retardation. *The American Journal of Human Genetics,* 76**,** 227-236.

JEONG, J. MAO, J. TENZEN, T. KOTTMANN, A.H. & MCMAHON, A.P. 2004. Hedgehog signaling in the neural crest cells regulates the patterning and growth of facial primordia. *Genes & Development,* 18**,** 937-951.

JEZEWSKI, P.A. VIEIRA, A.R. NISHIMURA, C. LUDWIG, B. JOHNSON, M. O'BRIEN, S.E. DAACK-HIRSCH, S. SCHULTZ, R.E. WEBER, A. NEPOMUCENA, B. ROMITTI, P.A. CHRISTENSEN, K. ORIOLI, I.M. CASTILLA, E.E. MACHIDA, J. NATSUME, N. & MURRAY, J.C. 2003. Complete sequencing shows a role for MSX1 in non-syndromic cleft lip and palate. *Journal of Medical Genetics,* 40**,** 399-407.

JIANG R, B.J.O., LIDRAL A.C. 2006. Development of the upper lip: Morphogenetic and molecular mechanisms. *Developmental Dynamics,* 235**,** 1152-1166.

JOHNSTON, M.C. & BRONSKY, P.T. 1995. Prenatal craniofacial development: new insights on normal and abnormal mechanisms. *Critical Reviews in Oral Biology and Medicine,* 6**,** 25-79.

JURILOFF, D.M. HARRIS, M.J. & BROWN, C.J. 2001. Unravelling the complex genetics of cleft lip in the mouse model. *Mammalian Genome,* 12**,** 426-435.

KALSCHEUER, V.M. FREUDE, K. MUSANTE, L. JENSEN, L.R. YNTEMA, H.G. GECZ, J. SEFIANI, A. HOFFMANN, K. MOSER, B. HAAS, S. GUROK, U. HAESLER, S. ARANDA, B. NSHEDJAN, A. TZSCHACH, A. HARTMANN, N. ROLOFF, T.C. SHOICHET, S. HAGENS, O. TAO, J. VAN BOKHOVEN, H. TURNER, G. CHELLY, J. MORAINE, C. FRYNS, J.P. NUBER, U. HOELTZENBEIN, M. SCHARFF, C. SCHERTHAN, H. LENZNER, S. HAMEL, B.C. SCHWEIGER, S. & ROPERS, H.H. 2003. Mutations in the polyglutamine binding protein 1 gene cause X-linked mental retardation. *Nature Genetics,* 35**,** 313-315.

KAMAK, H. & CELIKOGLU, M. 2012. Facial soft tissue thickness among skeletal malocclusions: is there a difference? *Korean Journal of Orthodontics,* 42**,** 23-31.

KAU, C.H. & RICHMOND, S. 2008. Three-dimensional analysis of facial morphology surface changes in untreated children from 12 to 14 years of age. *American Journal of Orthodontics and Dentofacial Orthopedics,* 134**,** 751-760.

KAU, C.H. & RICHMOND, S. 2010. *Three-Dimensional Imaging for Orthodontics and Maxillofacial Surgery*, Wiley Blackwell.

KAU, C.H. RICHMOND, S. PALOMO, J.M. & HANS, M.G. 2005a. Three-dimensional cone beam computerized tomography in orthodontics. *Journal of Orthodontics,* 32**,** 282-293.

KAU, C.H. RICHMOND, S. SAVIO, C. & MALLORIE, C. 2006. Measuring adult facial morphology in three dimensions. *The Angle Orthodontist* 76**,** 773-778.

KAU, C.H. RICHMOND, S. ZHUROV, A.I. KNOX, J. CHESTNUTT, I. HARTLES, F. & PLAYLE, R. 2005b. Reliability of measuring facial morphology with a 3-dimensional laser scanning system. *American Journal of Orthodontics and Dentofacial Orthopedics,* 128**,** 424-430.

KENNEDY, T.E. SERAFINI, T. DE LA TORRE, J.R. & TESSIER-LAVIGNE, M. 1994. Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. *Cell,* 78**,** 425-435.

KERNAHAN, D.A. DADO, D.V. & BAUER, B.S. 1984. The anatomy of the orbicularis oris muscle in unilateral cleft lip based on a three-dimensional histologic reconstruction. *Plastic and Reconstructive Surgery,* 73**,** 875-881.

KERNAHAN, D.A. & STARK, R.B. 1958. A new classification for cleft lip and cleft palate. *Plastic and Reconstructive Surgery and the Transplantation Bulletin,* 22**,** 435-441.

KHAMBAY, B. NEBEL, J.C. BOWMAN, J. WALKER, F. HADLEY, D.M. & AYOUB, A. 2002. 3D stereophotogrammetric image superimposition onto 3D CT scan images: the future of orthognathic surgery. A pilot study. *The International Journal of Adult Orthodontics and Orthognathic Surgery,* 17**,** 331-341.

KISHI, N. TANAKA, S. IIDA, S. & KOGO, M. 2012. The morphological features and developmental changes of the philtral dimple: a guide to surgical intervention in cases of cleft lip. *Journal of Cranio-Maxillo-Facial Surgery,* 40**,** 215-222.

KITSIOU-TZELI, S. KOLIALEXI, A. FRYSSIRA, H. GALLA-VOUMVOURAKI, A. SALAVOURA, K. KANARIOU, M. TSANGARIS, G.T. KANAVAKIS, E. & MAVROU, A. 2004. Detection of 22q11.2 deletion among 139 patients with Di George/Velocardiofacial syndrome features. *In Vivo,* 18**,** 603-608.

KLINGENBERG, C. 2013. Visualizations in geometric morphometrics: how to read and how to make graphs showing shape changes. *Hystrix, the Italian Journal of Mammalogy,* 24**,** 15-24.

KLOTZ, C.M. WANG, X. DESENSI, R.S. GRUBS, R.E. COSTELLO, B.J. & MARAZITA, M.L. 2010. Revisiting the recurrence risk of nonsyndromic cleft lip with or without cleft palate. *American Journal of Medical Genetics Part A,* 152A**,** 2697-2702.

KONDO, S. SCHUTTE, B.C. RICHARDSON, R.J. BJORK, B.C. KNIGHT, A.S. WATANABE, Y. HOWARD, E. FERREIRA DE LIMA, R.L.L. DAACK-HIRSCH, S. SANDER, A. MCDONALD-MCGINN, D.M. ZACKAI, E.H. LAMMER, E.J. AYLSWORTH, A.S. ARDINGER, H.H. LIDRAL, A.C. POBER, B.R. MORENO, L. ARCOS-BURGOS, M. VALENCIA, C. HOUDAYER, C. BAHUAU, M. MORETTI-FERREIRA, D. RICHIERI-COSTA, A. DIXON, M.J. & MURRAY, J.C. 2002. Mutations in IRF6 cause Van der Woude and popliteal pterygium syndromes. *Nature Genetics,* 32**,** 285-289.

KOOK, M.S. JUNG, S. PARK, H.J. OH, H.K. RYU, S.Y. CHO, J.H. LEE, J.S. YOON, S.J. KIM, M.S. & SHIN, H.K. 2014. A comparison study of different facial soft tissue analysis methods. *Journal of Cranio-Maxillo-Facial Surgery,* 42**,** 648-656.

KOOLEN, D.A. HERBERGS, J. VELTMAN, J.A. PFUNDT, R. VAN BOKHOVEN, H. STROINK, H. SISTERMANS, E.A. BRUNNER, H.G. GEURTS VAN KESSEL, A. & DE VRIES, B.B. 2006. Holoprosencephaly and preaxial polydactyly associated with a 1.24 Mb duplication encompassing FBXW11 at 5q35.1. *Journal of Human Genetics,* 51**,** 721-726.

KRAKOW, D. ROBERTSON, S.P. KING, L.M. MORGAN, T. SEBALD, E.T. BERTOLOTTO, C. WACHSMANN-HOGIU, S. ACUNA, D. SHAPIRO, S.S. TAKAFUTA, T. AFTIMOS, S. KIM, C.A. FIRTH, H. STEINER, C.E. CORMIER-DAIRE, V. SUPERTI-FURGA, A. BONAFE, L. GRAHAM, J.M., JR. GRIX, A. BACINO, C.A. ALLANSON, J. BIALER, M.G. LACHMAN, R.S. RIMOIN, D.L. & COHN, D.H. 2004. Mutations in the gene encoding filamin B disrupt vertebral segmentation, joint formation and skeletogenesis. *Nature Genetics,* 36**,** 405-410.

KRANTZ, I.D. MCCALLUM, J. DESCIPIO, C. KAUR, M. GILLIS, L.A. YAEGER, D. JUKOFSKY, L. WASSERMAN, N. BOTTANI, A. MORRIS, C.A. NOWACZYK, M.J. TORIELLO, H. BAMSHAD, M.J. CAREY, J.C. RAPPAPORT, E. KAWAUCHI, S. LANDER, A.D. CALOF, A.L. LI, H.H. DEVOTO, M. & JACKSON, L.G. 2004. Cornelia de Lange syndrome is caused by mutations in NIPBL, the human homolog of Drosophila melanogaster Nipped-B. *Nature Genetics,* 36**,** 631-635.

KRIENS, O. 1991. Data-objective diagnosis of infant cleft lip, alveolus, and palate. Morphologic data guiding understanding and treatment concepts. *The Cleft Palate Craniofacial Journal* 28**,** 157-168.

KRUGLYAK, L. & NICKERSON, D.A. 2001. Variation is the spice of life. *Nature Genetics* 27**,** 234-236.

KUROSAKA, H. 2015. The Roles of Hedgehog Signaling in Upper Lip Formation. *BioMed Research International,* 2015**,** 901041.

KUROSAKA, H. IULIANELLA, A. WILLIAMS, T. & TRAINOR, P.A. 2014. Disrupting hedgehog and WNT signaling interactions promotes cleft lip pathogenesis. *The Journal of Clinical Investigation,* 124**,** 1660-1671.

KUROSAKA, H. TRAINOR, P.A. LEROUX-BERGER, M. & IULIANELLA, A. 2015. Cranial nerve development requires co-ordinated Shh and canonical Wnt signaling. *PLoS One,* 10**,** e0120821.

LAN, Y. RYAN, R.C. ZHANG, Z. BULLARD, S.A. BUSH, J.O. MALTBY, K.M. LIDRAL, A.C. & JIANG, R. 2006. Expression of Wnt9b and activation of canonical Wnt signaling during midfacial morphogenesis in mice. *Developmental Dynamics,* 235**,** 1448-1454.

LANDIS, J.R. & KOCH, G.G. 1977. The measurement of observer agreement for categorical data. *Biometrics,* 33**,** 159-174.

LARSEN, A.K. MIKKELSEN, D.B. HERTZ, J.M. & BYGUM, A. 2014. Manifestations of Gorlin-Goltz syndrome. *Danish Medical Journal,* 61**,** 1-5.

LATHAM, R.A. & DEATON, T.G. 1976. The structural basis of the philtrum and the contour of the vermilion border: a study of the musculature of the upper lip. *Journal of Anatomy,* 121**,** 151-160.

LAUE, K. POGODA, H.M. DANIEL P , B. VAN HAERINGEN, A. ALANAY, Y. VON AMELN, S. RACHWALSKI, M. MORGAN, T. GRAY M , J. BREUNING M , H. SAWYER G , M. SUTHERLAND-SMITH A , J. NIKKELS P , G. KUBISCH, C. BLOCH, W. WOLLNIK, B. HAMMERSCHMIDT, M. & ROBERTSON S , P. 2011. Craniosynostosis and

Multiple Skeletal Anomalies in Humans and Zebrafish Result from a Defect in the Localized Degradation of Retinoic Acid. *The American Journal of Human Genetics,* 89**,** 595-606.

LAUMONNIER, F. HOLBERT, S. RONCE, N. FARAVELLI, F. LENZNER, S. SCHWARTZ, C.E. LESPINASSE, J. VAN ESCH, H. LACOMBE, D. GOIZET, C. PHAN-DINH TUY, F. VAN BOKHOVEN, H. FRYNS, J.P. CHELLY, J. ROPERS, H.H. MORAINE, C. HAMEL, B.C. & BRIAULT, S. 2005. Mutations in PHF8 are associated with X linked mental retardation and cleft lip/cleft palate. *Journal of Medical Genetics,* 42**,** 780-786.

LAURIN, M. HUBER, J. PELLETIER, A. HOUALLA, T. PARK, M. FUKUI, Y. HAIBE-KAINS, B. MULLER, W.J. & COTE, J.F. 2013. Rac-specific guanine nucleotide exchange factor DOCK1 is a critical regulator of HER2-mediated breast cancer metastasis. *Proceedings of the National Academy of Sciences,* 110**,** 7434-7439.

LEBOW, M.R. & SAWIN, P.B. 1941. Inheritance of human facial features: A Pedigree Study Involving Length of Face, Prominent Ears and Chin Cleft. *Journal of Heredity,* 32**,** 127-132.

LEOYKLANG, P. SIRIWAN, P. & SHOTELERSUK, V. 2006. A mutation of the p63 gene in non-syndromic cleft lip. *Journal of Medical Genetics,* 43**,** e28.

LESLIE, E.J. CARLSON, J.C. COOPER, M.E. CHRISTENSEN, K. WEINBERG, S.M. & MARAZITA, M.L. 2016. Exploring Subclinical Phenotypic Features in Twin Pairs Discordant for Cleft Lip and Palate. *The Cleft Palate Craniofacial Journal*

LETTICE, L.A. HEANEY, S.J. PURDIE, L.A. LI, L. DE BEER, P. OOSTRA, B.A. GOODE, D. ELGAR, G. HILL, R.E. & DE GRAAFF, E. 2003. A long-range Shh enhancer regulates expression in the developing limb and fin and is associated with preaxial polydactyly. *Human Molecular Genetics,* 12**,** 1725-1735.

LEVAILLANT, J.M. MOEGLIN, D. ZOUITEN, K. BUCOURT, M. BURGLEN, L. SOUPRE, V. BAUMANN, C. JAQUEMONT, M.L. TOURAINE, R. PICARD, A. VUILLARD, E. BELARBI, N. OURY, J.F. VERLOES, A. VAZQUEZ, M.P. LABRUNE, P. DELEZOIDE, A.L. & GERARD-BLANLUET, M. 2009. Binder phenotype: clinical and etiological heterogeneity of the so-called Binder maxillonasal dysplasia in prenatally diagnosed cases, and review of the literature. *Prenatal Diagnosis,* 29**,** 140-150.

LEW, K.K. FOONG, W.C. & LOH, E. 1993. Malocclusion prevalence in an ethnic Chinese population. *Australian Dental Journal,* 38**,** 442-449.

LI, H. WANG, C.Y. WANG, J.X. WU, G.S. YU, P. YAN, X.Y. CHEN, Y.G. ZHAO, L.H. & ZHANG, Y.P. 2009. Mutation analysis of a large Chinese pedigree with congenital preaxial polydactyly. *European Journal of Human Genetics,* 17**,** 604-610.

LI, Y. WILLER, C.J. DING, J. SCHEET, P. & ABECASIS, G.R. 2010. MaCH: using sequence and genotype data to estimate haplotypes and unobserved genotypes. *Genetic Epidemiology,* 34**,** 816-834.

LIM, S. & KALDIS, P. 2013. Cdks, cyclins and CKIs: roles beyond cell cycle regulation. *Development,* 140**,** 3079-3093.

LITTLE, J. CARDY, A. & MUNGER, R.G. 2004. Tobacco smoking and oral clefts: a meta-analysis. *Bulletin of the World Health Organisation,* 82**,** 213-218.

LIU, F. VAN DER LIJN, F. SCHURMANN, C. ZHU, G. CHAKRAVARTY, M.M. HYSI, P.G. WOLLSTEIN, A. LAO, O. DE BRUIJNE, M. IKRAM, M.A. VAN DER LUGT, A. RIVADENEIRA, F. UITTERLINDEN AÉ, G. HOFMAN, A. NIESSEN, W.J. HOMUTH, G. DE ZUBICARAY, G. MCMAHON, K.L. THOMPSON, P.M. DABOUL, A. PULS, R. HEGENSCHEID, K. BEVAN, L. PAUSOVA, Z. MEDLAND, S.E. MONTGOMERY, G.W. WRIGHT, M.J. WICKING, C. BOEHRINGER, S. SPECTOR, T.D. PAUS, T. MARTIN,

N.G. BIFFAR, R. & KAYSER, M. 2012a. A Genome-Wide Association Study Identifies Five Loci Influencing Facial Morphology in Europeans. *PLoS Genet,* 8.

LIU, F. VAN DER LIJN, F. SCHURMANN, C. ZHU, G. CHAKRAVARTY, M.M. HYSI, P.G. WOLLSTEIN, A. LAO, O. DE BRUIJNE, M. IKRAM, M.A. VAN DER LUGT, A. RIVADENEIRA, F. UITTERLINDEN, A.G. HOFMAN, A. NIESSEN, W.J. HOMUTH, G. DE ZUBICARAY, G. MCMAHON, K.L. THOMPSON, P.M. DABOUL, A. PULS, R. HEGENSCHEID, K. BEVAN, L. PAUSOVA, Z. MEDLAND, S.E. MONTGOMERY, G.W. WRIGHT, M.J. WICKING, C. BOEHRINGER, S. SPECTOR, T.D. PAUS, T. MARTIN, N.G. BIFFAR, R. & KAYSER, M. 2012b. A genome-wide association study identifies five loci influencing facial morphology in Europeans. *PLoS Genet,* 8**,** e1002932.

LIU, F. VAN DER LIJN, F. SCHURMANN, C. ZHU, G. CHAKRAVARTY, M.M. HYSI, P.G. WOLLSTEIN, A. LAO, O. DE BRUIJNE, M. IKRAM, M.A. VAN DER LUGT, A. RIVADENEIRA, F. UITTERLINDEN, A.G. HOFMAN, A. NIESSEN, W.J. HOMUTH, G. DE ZUBICARAY, G. MCMAHON, K.L. THOMPSON, P.M. DABOUL, A. PULS, R. HEGENSCHEID, K. BEVAN, L. PAUSOVA, Z. MEDLAND, S.E. MONTGOMERY, G.W. WRIGHT, M.J. WICKING, C. BOEHRINGER, S. SPECTOR, T.D. PAUS, T. MARTIN, N.G. BIFFAR, R. & KAYSER, M. 2012c. A genome-wide association study identifies five loci influencing facial morphology in Europeans. *PLoS Genetics,* 8**,** e1002932.

LIU, S. AN, H. LI, N. YU, Y. LIN, N. WAN, T. ZHANG, M. WANG, W. & CAO, X. 2003. Cloning and identification of a novel human ubiquitin-like protein, DC-UbP, from dendritic cells. *Biochemical and Biophysical Research Communications,* 300**,** 800-805.

LIU, W. SELEVER, J. MURALI, D. SUN, X. BRUGGER, S.M. MA, L. SCHWARTZ, R.J. MAXSON, R. FURUTA, Y. & MARTIN, J.F. 2005. Threshold-specific requirements for Bmp4 in mandibular development. *Developmental Biology,* 283**,** 282-293.

LOEYS, B.L. CHEN, J. NEPTUNE, E.R. JUDGE, D.P. PODOWSKI, M. HOLM, T. MEYERS, J. LEITCH, C.C. KATSANIS, N. SHARIFI, N. XU, F.L. MYERS, L.A. SPEVAK, P.J. CAMERON, D.E. DE BACKER, J. HELLEMANS, J. CHEN, Y. DAVIS, E.C. WEBB, C.L. KRESS, W. COUCKE, P. RIFKIN, D.B. DE PAEPE, A.M. & DIETZ, H.C. 2005. A syndrome of altered cardiovascular, craniofacial, neurocognitive and skeletal development caused by mutations in TGFBR1 or TGFBR2. *Nature Genetics* 37**,** 275-281.

LOPEZ, E. THAUVIN-ROBINET, C. REVERSADE, B. KHARTOUFI, N.E. DEVISME, L. HOLDER, M. ANSART-FRANQUET, H. AVILA, M. LACOMBE, D. KLEINFINGER, P. KAORI, I. TAKANASHI, J. LE MERRER, M. MARTINOVIC, J. NOEL, C. SHBOUL, M. HO, L. GUVEN, Y. RAZAVI, F. BURGLEN, L. GIGOT, N. DARMENCY-STAMBOUL, V. THEVENON, J. ARAL, B. KAYSERILI, H. HUET, F. LYONNET, S. LE CAIGNEC, C. FRANCO, B. RIVIERE, J.B. FAIVRE, L. & ATTIE-BITACH, T. 2014. C5orf42 is the major gene responsible for OFD syndrome type VI. *Human Genetics,* 133**,** 367-377.

LUDWIG, K.U. BOHMER, A.C. RUBINI, M. MOSSEY, P.A. HERMS, S. NOWAK, S. REUTTER, H. ALBLAS, M.A. LIPPKE, B. BARTH, S. PAREDES-ZENTENO, M. MUNOZ-JIMENEZ, S.G. ORTIZ-LOPEZ, R. KREUSCH, T. HEMPRICH, A. MARTINI, M. BRAUMANN, B. JAGER, A. POTZSCH, B. MOLLOY, A. PETERLIN, B. HOFFMANN, P. NOTHEN, M.M. ROJAS-MARTINEZ, A. KNAPP, M. STEEGERS-THEUNISSEN, R.P. & MANGOLD, E. 2014. Strong association of variants around FOXE1 and orofacial clefting. *Journal of Dental Research,* 93**,** 376-381.

LUDWIG, K.U. MANGOLD, E. HERMS, S. NOWAK, S. REUTTER, H. PAUL, A. BECKER, J. HERBERZ, R. ALCHAWA, T. NASSER, E. BÖHMER, A.C. MATTHEISEN, M. ALBLAS, M.A. BARTH, S. KLUCK, N. LAUSTER, C. BRAUMANN, B. REICH, R.H. HEMPRICH, A. PÖTZSCH, S. BLAUMEISER, B. DARATSIANOS, N. KREUSCH, T. MURRAY, J.C. MARAZITA, M.L. RUCZINSKI, I. SCOTT, A.F. BEATY, T.H. KRAMER, F.-J. WIENKER, T.F. STEEGERS-THEUNISSEN, R.P. RUBINI, M. MOSSEY, P.A. HOFFMANN, P. LANGE, C. CICHON, S. PROPPING, P. KNAPP, M. & NÖTHEN, M.M. 2012. Genome-wide meta-analyses of nonsyndromic cleft lip with or without cleft palate identify six new risk loci. *Nature Genetics,* 44**,** 968-971.

MAMANDRAS, A.H. 1984. Growth of lips in two dimensions: A serial cephalometric study. *American Journal of Orthodontics,* 86**,** 61-66.

MAMANDRAS, A.H. 1988. Linear changes of the maxillary and mandibular lips. *American Journal of Orthodontics and Dentofacial Orthopedics,* 94**,** 405-410.

MANGOLD, E. LUDWIG, K.U. BIRNBAUM, S. BALUARDO, C. FERRIAN, M. HERMS, S. REUTTER, H. DE ASSIS, N.A. CHAWA, T.A. MATTHEISEN, M. STEFFENS, M. BARTH, S. KLUCK, N. PAUL, A. BECKER, J. LAUSTER, C. SCHMIDT, G. BRAUMANN, B. SCHEER, M. REICH, R.H. HEMPRICH, A. POTZSCH, S. BLAUMEISER, B. MOEBUS, S. KRAWCZAK, M. SCHREIBER, S. MEITINGER, T. WICHMANN, H.E. STEEGERS-THEUNISSEN, R.P. KRAMER, F.J. CICHON, S. PROPPING, P. WIENKER, T.F. KNAPP, M. RUBINI, M. MOSSEY, P.A. HOFFMANN, P. & NOTHEN, M.M. 2010. Genome-wide association study identifies two susceptibility loci for nonsyndromic cleft lip with or without cleft palate. *Nature Genetics,* 42**,** 24-26.

MANGOLD, E. LUDWIG, K.U. & NÖTHEN, M.M. 2011. Breakthroughs in the genetics of orofacial clefting. *Trends in Molecular Medicine,* 17**,** 725-733.

MANGOLD, E. REUTTER, H. BIRNBAUM, S. WALIER, M. MATTHEISEN, M. HENSCHKE, H. LAUSTER, C. SCHMIDT, G. SCHIEFKE, F. REICH, R.H. SCHEER, M. HEMPRICH, A. MARTINI, M. BRAUMANN, B. KRIMMEL, M. OPITZ, C. LENZ, J.H. KRAMER, F.J. WIENKER, T.F. NOTHEN, M.M. & DIAZ LACAVA, A. 2009. Genome-wide linkage scan of nonsyndromic orofacial clefting in 91 families of central European origin. *American Journal of Medical Genetics Part A,* 149A**,** 2680-2694.

MANOLIO, T.A. & COLLINS, F.S. 2009. The HapMap and Genome-Wide Association Studies in Diagnosis and Therapy. *Annual Review of Medicine,* 60**,** 443-456.

MARAZITA, M.L. GOLDSTEIN, A.M. SMALLEY, S.L. SPENCE, M.A. & RAO, D.C. 1986. Cleft lip with or without cleft palate: Reanalysis of a three-generation family study from England. *Genetic Epidemiology,* 3**,** 335-342.

MARAZITA, M.L. MURRAY, J.C. LIDRAL, A.C. ARCOS-BURGOS, M. COOPER, M.E. GOLDSTEIN, T. MAHER, B.S. DAACK-HIRSCH, S. SCHULTZ, R. MANSILLA, M.A. FIELD, L.L. LIU, Y.E. PRESCOTT, N. MALCOLM, S. WINTER, R. RAY, A. MORENO, L. VALENCIA, C. NEISWANGER, K. WYSZYNSKI, D.F. BAILEY-WILSON, J.E. ALBACHA-HEJAZI, H. BEATY, T.H. MCINTOSH, I. HETMANSKI, J.B. TUNCBILEK, G. EDWARDS, M. HARKIN, L. SCOTT, R. & RODDICK, L.G. 2004. Meta-analysis of 13 genome scans reveals multiple cleft lip/palate genes with novel loci on 9q21 and 2q32-35. *The American Journal of Human Genetics,* 75**,** 161-173.

MATSUI, M. & KLINGENSMITH, J. 2014. Multiple tissue-specific requirements for the BMP antagonist Noggin in development of the mammalian craniofacial skeleton. *Developmental Biology,* 392**,** 168-181.

MAYES, K. QIU, Z. ALHAZMI, A. & LANDRY, J.W. 2014. ATP-Dependent Chromatin Remodeling Complexes as Novel Targets for Cancer Therapy. *Advances in Cancer Research,* 121**,** 183-233.

MCBRIDE, W.A. MCINTYRE, G.T. CARROLL, K. & MOSSEY, P.A. 2016. Subphenotyping and Classification of Orofacial Clefts: Need for Orofacial Cleft Subphenotyping Calls for Revised Classification. *The Cleft Palate Craniofacial Journal* 53**,** 539-549.

MCINTYRE, G.T. & MILLETT, D.T. 2006. Lip shape and position in Class II division 2 malocclusion. *The Angle Orthodontist,* 76**,** 739-744.

MEE, L. HONKALA, H. KOPRA, O. VESA, J. FINNILA, S. VISAPAA, I. SANG, T.K. JACKSON, G.R. SALONEN, R. KESTILA, M. & PELTONEN, L. 2005. Hydrolethalus syndrome is caused by a missense mutation in a novel gene HYLS1. *Human Molecular Genetics,* 14**,** 1475-1488.

MEHES, K. 1981a. Normal values for philtral length, oral intercommissural distance, and sternal length in normal newborn infants *J Craniofac Genet Dev Biol* 1**,** 213–216.

MEHES, K. 1981b. Normal values of philtral length, oral intercommissural distance, and sternal length in newborn infants. *Journal of Craniofacial Genetics and Developmental Biology,* 1**,** 213-216.

MEHTA, L. DUCKETT, D.P. & YOUNG, I.D. 1987. Behaviour disorder in monosomy 10qter. *Journal of Medical Genetics,* 24**,** 185-186.

MELMAN, Y.F. UM, S.Y. KRUMERMAN, A. KAGAN, A. & MCDONALD, T.V. 2004. KCNE1 Binds to the KCNQ1 Pore to Regulate Potassium Channel Activity. *Neuron,* 42**,** 927-937.

MERRETT, S.J. DRAGE, N.A. & DURNING, P. 2009. Cone beam computed tomography: a useful tool in orthodontic diagnosis and treatment planning. *Journal of Orthodontics,* 36**,** 202-210.

MERRILL, B.J. PASOLLI, H.A. POLAK, L. RENDL, M. GARCIA-GARCIA, M.J. ANDERSON, K.V. & FUCHS, E. 2004. Tcf3: a transcriptional regulator of axis induction in the early embryo. *Development,* 131**,** 263-274.

MIETTINEN, P.J. CHIN, J.R. SHUM, L. SLAVKIN, H.C. SHULER, C.F. DERYNCK, R. & WERB, Z. 1999. Epidermal growth factor receptor function is necessary for normal craniofacial development and palate closure. *Nature Genetics* 22**,** 69-73.

MILLARD, D.R. 1958. A radical rotation in single harelip. *American Journal of Surgery,* 95**,** 318-324.

MILLER, S.F. WEINBERG, S.M. NIDEY, N.L. DEFAY, D.K. MARAZITA, M.L. WEHBY, G.L. & MORENO URIBE, L.M. 2014a. Exploratory genotype-phenotype correlations of facial form and asymmetry in unaffected relatives of children with non-syndromic cleft lip and/or palate. *Journal of Anatomy,* 224**,** 688-709.

MILLER, S.F. WEINBERG, S.M. NIDEY, N.L. DEFAY, D.K. MARAZITA, M.L. WEHBY, G.L. & URIBE, L.M.M. 2014b. Exploratory genotype–phenotype correlations of facial form and asymmetry in unaffected relatives of children with non-syndromic cleft lip and/or palate. *Journal of Anatomy,* 224**,** 688-709.

MILLICOVSKY P.J, J.M.C. 1981. Active role of embryonic facial epithelium: new evidence of cellular events in morphogenesis. *J Embryol Exp Morphol,* 63**,** 53-66.

MILUNSKY, J.M. MAHER, T.A. ZHAO, G. ROBERTS, A.E. STALKER, H.J. ZORI, R.T. BURCH, M.N. CLEMENS, M. MULLIKEN, J.B. SMITH, R. & LIN, A.E. 2008. TFAP2A mutations result in branchio-oculo-facial syndrome. *The American Journal of Human Genetics,* 82**,** 1171-1177.

MITCHELL, L.E. MURRAY, J.C. O'BRIEN, S. & CHRISTENSEN, K. 2003. Retinoic acid receptor alpha gene variants, multivitamin use, and liver intake as risk factors

for oral clefts: a population-based case-control study in Denmark, 1991-1994. *American Journal of Epidemiology,* 158**,** 69-76.

MITCHELL, L.E. & RISCH, N. 1992. Mode of inheritance of nonsyndromic cleft lip with or without cleft palate: a reanalysis. *The American Journal of Human Genetics,* 51**,** 323-332.

MO, W. ZHANG, J. LI, X. MENG, D. GAO, Y. YANG, S. WAN, X. ZHOU, C. GUO, F. HUANG, Y. AMENTE, S. AVVEDIMENTO, E.V. XIE, Y. & LI, Y. 2013. Identification of novel AR-targeted microRNAs mediating androgen signalling through critical pathways to regulate cell viability in prostate cancer. *PLoS One,* 8**,** e56592.

MOOTOOSAMY, R.C. & DIETRICH, S. 2002. Distinct regulatory cascades for head and trunk myogenesis. *Development,* 129**,** 573-583.

MORGAN, N.V. BRUETON, L.A. COX, P. GREALLY, M.T. TOLMIE, J. PASHA, S. ALIGIANIS, I.A. VAN BOKHOVEN, H. MARTON, T. AL-GAZALI, L. MORTON, J.E. OLEY, C. JOHNSON, C.A. TREMBATH, R.C. BRUNNER, H.G. & MAHER, E.R. 2006. Mutations in the embryonal subunit of the acetylcholine receptor (CHRNG) cause lethal and Escobar variants of multiple pterygium syndrome. *The American Journal of Human Genetics,* 79**,** 390-395.

MORI, A. NAKAJIMA, T. KANEKO, T. SAKUMA, H. & AOKI, Y. 2005. Analysis of 109 Japanese children's lip and nose shapes using 3-dimensional digitizer. *British Journal of Plastic Surgery,* 58**,** 318-329.

MORLEO, M. WOOLFALL, K. DEDMAN, D. MUKHERJEE, R. BELLIS, M.A. & COOK, P.A. 2011. Under-reporting of foetal alcohol spectrum disorders: an analysis of hospital episode statistics. *BMC Pediatrics,* 11**,** 14.

MOSBY 2009. *Medical Dictionary*.

MOSSEY, P.A. BATRA, P. & MCINTYRE, G.T. 2010. The parental dentocraniofacial phenotype-an orofacial clefting microform. *The Cleft Palate Craniofacial Journal* 47**,** 22-34.

MOSSEY, P.A. LITTLE, J. MUNGER, R.G. DIXON, M.J. & SHAW, W.C. 2009. Cleft lip and palate. *The Lancet,* 374**,** 1773-1785.

MOSSEY, P.A. MCCOLL, J.H. & STIRRUPS, D.R. 1997. Differentiation between cleft lip with or without cleft palate and isolated cleft palate using parental cephalometric parameters. *The Cleft Palate Craniofacial Journal* 34**,** 27-35.

MOSTOWSKA, A. HOZYASZ, K.K. & JAGODZINSKI, P.P. 2006. Maternal MTR genotype contributes to the risk of non-syndromic cleft lip and palate in the Polish population. *Clinical Genetics,* 69**,** 512-517.

MULLIKEN, J.B. PENSLER, J.M. & KOZAKEWICH, H.P. 1993. The anatomy of Cupid's bow in normal and cleft lip. *Plastic and Reconstructive Surgery,* 92**,** 395-403; discussion 404.

NAGALAXMI, V. UGRAPPA, S. M, N.J. CH, L. MALOTH, K.N. & KODANGAL, S. 2014. Cheiloscopy, Palatoscopy and Odontometrics in Sex Prediction and Dis-crimination - a Comparative Study. *The Open Dentistry Journal,* 8**,** 269-279.

NAM, K.W. PARK, J. KIM, I.Y. & KIM, K.G. 2012. Application of Stereo-Imaging Technology to Medical Field. *Healthcare Informatics Research,* 18**,** 158-163.

NAMNOUM, J.D. HISLEY, K.C. GRAEPEL, S. HUTCHINS, G.N. & VANDER KOLK, C.A. 1997. Three-dimensional reconstruction of the human fetal philtrum. *Annals of Plastic Surgery,* 38**,** 202-208.

NANDA, R.S. MENG, H. KAPILA, S. & GOORHUIS, J. 1990. Growth changes in the soft tissue facial profile. *The Angle Orthodontist,* 60**,** 177-190.

NCBI. 2015. Available: http://www.ncbi.nlm.nih.gov/projects/SNP/snp_summary.cgi.

NEISWANGER, K. WALKER, K. KLOTZ, C.M. COOPER, M.E. BARDI, K.M. BRANDON, C.A. WEINBERG, S.M. VIEIRA, A.R. MARTIN, R.A. CZEIZEL, A.E. CASTILLA, E.E. POLETTA, F.A. & MARAZITA, M.L. 2009. Whorl patterns on the lower lip are associated with nonsyndromic cleft lip with or without cleft palate. *American Journal of Medical Genetics Part A,* 149A**,** 2673-2679.

NEISWANGER, K. WEINBERG, S.M. ROGERS, C.R. BRANDON, C.A. COOPER, M.E. BARDI, K.M. DELEYIANNIS, F.W. RESICK, J.M. BOWEN, A. MOONEY, M.P. DE SALAMANCA, J.E. GONZALEZ, B. MAHER, B.S. MARTIN, R.A. & MARAZITA, M.L. 2007. Orbicularis oris muscle defects as an expanded phenotypic feature in nonsyndromic cleft lip with or without cleft palate. *American Journal of Medical Genetics Part A,* 143A**,** 1143-1149.

NEUBUSER, A. PETERS, H. BALLING, R. & MARTIN, G.R. 1997. Antagonistic interactions between FGF and BMP signaling pathways: a mechanism for positioning the sites of tooth formation. *Cell,* 90**,** 247-255.

NEVADO, J. MERGENER, R. PALOMARES-BRALO, M. SOUZA, K.R. VALLESPÍN, E. MENA, R. MARTÍNEZ-GLEZ, V. MORI, M. SANTOS, F. GARCÍA-MIÑAUR, S. GARCÍA-SANTIAGO, F. MANSILLA, E. FERNÁNDEZ, L. DE TORRES MÍ, L. RIEGEL, M. & LAPUNZINA, P. 2014. New microdeletion and microduplication syndromes: A comprehensive review. *Genetics and Molecular Biology,* 37**,** 210-219.

NG, D. THAKKER, N. CORCORAN, C.M. DONNAI, D. PERVEEN, R. SCHNEIDER, A. HADLEY, D.W. TIFFT, C. ZHANG, L. WILKIE, A.O. VAN DER SMAGT, J.J. GORLIN, R.J. BURGESS, S.M. BARDWELL, V.J. BLACK, G.C. & BIESECKER, L.G. 2004. Oculofaciocardiodental and Lenz microphthalmia syndromes result from distinct classes of mutations in BCOR. *Nature Genetics,* 36**,** 411-416.

NG, S.B. BIGHAM, A.W. BUCKINGHAM, K.J. HANNIBAL, M.C. MCMILLIN, M.J. GILDERSLEEVE, H.I. BECK, A.E. TABOR, H.K. COOPER, G.M. MEFFORD, H.C. LEE, C. TURNER, E.H. SMITH, J.D. RIEDER, M.J. YOSHIURA, K. MATSUMOTO, N. OHTA, T. NIIKAWA, N. NICKERSON, D.A. BAMSHAD, M.J. & SHENDURE, J. 2010. Exome sequencing identifies MLL2 mutations as a cause of Kabuki syndrome. *Nature Genetics,* 42**,** 790-793.

NIEMANN, S. 1993. Tetra-Amelia Syndrome. *In:* PAGON, R.A.ADAM, M.P.ARDINGER, H.H.WALLACE, S.E.AMEMIYA, A.BEAN, L.J.H.BIRD, T.D.FONG, C.T.MEFFORD, H.C.SMITH, R.J.H. & STEPHENS, K. (eds.) *GeneReviews(R).* Seattle WA: University of Washington, Seattle.

NOGUEIRA, J.M. HAWROT, K. SHARPE, C. NOBLE, A. WOOD, W.M. JORGE, E.C. GOLDHAMER, D.J. KARDON, G. & DIETRICH, S. 2015. The emergence of Pax7-expressing muscle stem cells during vertebrate head muscle development. *Frontiers in Aging Neuroscience,* 19**,** 62.

O'RAHILLY, R. 1972. Guide to the staging of human embryos. *Anat Anz,* 130**,** 556-559.

OFFICER, C.M. 2015. UK Chief Medical Officers' Alcohol Guidelines Review Summary of the proposed new guidelines. *In:* HEALTH, D.O. (ed.).

OHBAYASHI, N. & ETO, K. 1986. Relative contributions of the facial processes to facial development: a microsurgical assay. *Journal of Craniofacial Genetics and Developmental Biology,* 2**,** 41-44.

OHBAYASHI N., E.K. 1986. Relative contributions of the facial processes to facial development: a microsurgical assay. *J Craniofac Genet Dev Biol Suppl,* 2**,** 41-44.

OKAMOTO, N. HAYASHI, S. MASUI, A. KOSAKI, R. OGURI, I. HASEGAWA, T. IMOTO, I. MAKITA, Y. HATA, A. MORIYAMA, K. & INAZAWA, J. 2012. Deletion at

chromosome 10p11.23-p12.1 defines characteristic phenotypes with marked midface retrusion. *Journal of Human Genetics,* 57**,** 191-196.

OKANO, J. UDAGAWA, J. & SHIOTA, K. 2014. Roles of retinoic acid signaling in normal and abnormal development of the palate and tongue. *Congenital Anomalies,* 54**,** 69-76.

OLIVEIRA-COSTA, J.P. ZANETTI, J. OLIVEIRA, L.R. SOARES, F.A. RAMALHO, L.Z. SILVA RAMALHO, F. GARCIA, S.B. & RIBEIRO-SILVA, A. 2010. Significance of topoisomerase IIIbeta expression in breast ductal carcinomas: strong associations with disease-specific survival and metastasis. *Human Pathology,* 41**,** 1624-1630.

OLSZEWSKI, R. LIU, Y. DUPREZ, T. XU, T.M. & REYCHLER, H. 2009. Three-dimensional appearance of the lips muscles with three-dimensional isotropic MRI: in vivo study. *International Journal of Computer Assisted Radiology and Surgery,* 4**,** 349-352.

PARK-WYLLIE, L. MAZZOTTA, P. PASTUSZAK, A. MORETTI, M.E. BEIQUE, L. HUNNISETT, L. FRIESEN, M.H. JACOBSON, S. KASAPINOVIC, S. CHANG, D. DIAV-CITRIN, O. CHITAYAT, D. NULMAN, I. EINARSON, T.R. & KOREN, G. 2000. Birth defects after maternal exposure to corticosteroids: prospective cohort study and meta-analysis of epidemiological studies. *Teratology,* 62**,** 385-392.

PARKER, G.D. MARSHALL, D. ROSIN, P.L. DRAGE, N. RICHMOND, S. & JONES, D.K. 2013. A pitfall in the reconstruction of fibre ODFs using spherical deconvolution of diffusion MRI data. *NeuroImage,* 65**,** 433-448.

PATEL, N. ITAKURA, T. GONZALEZ, J.M., JR. SCHWARTZ, S.G. & FINI, M.E. 2013. GPR158, an orphan member of G protein-coupled receptor Family C: glucocorticoid-stimulated expression and novel nuclear role. *PLoS One,* 8**,** e57843.

PATEL, N. ITAKURA, T. JEONG, S. LIAO, C.P. ROY-BURMAN, P. ZANDI, E. GROSHEN, S. PINSKI, J. COETZEE, G.A. GROSS, M.E. & FINI, M.E. 2015. Expression and functional role of orphan receptor GPR158 in prostate cancer growth and progression. *PLoS One,* 10**,** e0117758.

PATERNOSTER, L. ZHUROV, A. TOMA, A.M. KEMP, J.P. ST POURCAIN, B. TIMPSON, N.J. MCMAHON, G. & EVANS, D.M. 2012. Genome-wide association study of three-dimensional facial morphology identifies a variant in PAX3 associated with nasion position. *The American Journal of Human Genetics,* 90**,** 478-485.

PECK, S. PECK, L. & KATAJA, M. 1992. Some vertical lineaments of lip position. *American Journal of Orthodontics and Dentofacial Orthopedics,* 101**,** 519-524.

PELIN, K. RIDANPAA, M. DONNER, K. WILTON, S. KRISHNARAJAH, J. LAING, N. KOLMERER, B. MILLEVOI, S. LABEIT, S. DE LA CHAPELLE, A. & WALLGREN-PETTERSON, C. 1997. Refined localisation of the genes for nebulin and titin on chromosome 2q allows the assignment of nebulin as a candidate gene for autosomal recessive nemaline myopathy. *European Journal of Human Genetics,* 5**,** 229-234.

PENG, S. TAN, J. HU, S. ZHOU, H. GUO, J. JIN, L. & TANG, K. 2013. Detecting Genetic Association of Common Human Facial Morphological Variation Using High Density 3D Image Registration. *PLoS Computational Biology,* 9.

PITHON, M.M. RODRIGUES RIBEIRO, D.L. LACERDA DOS SANTOS, R. LEITE DE SANTANA, C. & PEDROSA CRUZ, J.P. 2014. Soft tissue thickness in young north eastern Brazilian individuals with different skeletal classes. *Journal of Forensic and Legal Medicine,* 22**,** 115-120.

PLOOIJ, J.M. MAAL, T.J. HAERS, P. BORSTLAP, W.A. KUIJPERS-JAGTMAN, A.M. & BERGE, S.J. 2011. Digital three-dimensional image fusion processes for planning and evaluating orthodontics and orthognathic surgery. A systematic review. *International Journal of Oral and Maxillofacial Surgery,* 40**,** 341-352.

POPAT, H. RICHMOND, S. & DRAGE, N.A. 2010. New developments in: three-dimensional planning for orthognathic surgery. *Journal of Orthodontics,* 37**,** 62-71.

PRABHU, R.V. DINKAR, A.D. PRABHU, V.D. & RAO, P.K. 2012. Cheiloscopy: Revisited. *Journal of Forensic Dental Sciences,* 4**,** 47-52.

PROCHAZKOVA, J. & TOLAROVA, M. 1986. Craniofacial morphological features in parents of children with isolated cleft palate. *Acta Chirurgiae Plasticae,* 28**,** 194-204.

QUADERI, N.A. SCHWEIGER, S. GAUDENZ, K. FRANCO, B. RUGARLI, E.I. BERGER, W. FELDMAN, G.J. VOLTA, M. ANDOLFI, G. GILGENKRANTZ, S. MARION, R.W. HENNEKAM, R.C. OPITZ, J.M. MUENKE, M. ROPERS, H.H. & BALLABIO, A. 1997. Opitz G/BBB syndrome, a defect of midline development, is due to mutations in a new RING finger gene on Xp22. *Nature Genetics,* 17**,** 285-291.

QURESHI, S.F. ALI, A. ANANTHAPUR, V. JAYAKRISHNAN, M.P. CALAMBUR, N. THANGARAJ, K. & NALLARI, P. 2013. Novel mutations of KCNQ1 in Long QT syndrome. *Indian Heart Journal,* 65**,** 552-560.

RALSTON, S.H. GALWEY, N. MACKAY, I. ALBAGHA, O.M. CARDON, L. COMPSTON, J.E. COOPER, C. DUNCAN, E. KEEN, R. LANGDAHL, B. MCLELLAN, A. O'RIORDAN, J. POLS, H.A. REID, D.M. UITTERLINDEN, A.G. WASS, J. & BENNETT, S.T. 2005. Loci for regulation of bone mineral density in men and women identified by genome wide linkage scan: the FAMOS study. *Human Molecular Genetics,* 14**,** 943-951.

REARDON, W. WINTER, R.M. RUTLAND, P. PULLEYN, L.J. JONES, B.M. & MALCOLM, S. 1994. Mutations in the fibroblast growth factor receptor 2 gene cause Crouzon syndrome. *Nature Genetics,* 8**,** 98-103.

RICKETTS, R.M. 1968. Esthetics, environment, and the law of lip relation. *American Journal of Orthodontics,* 54**,** 272-289.

RIED, J.S. JEFF, M.J. CHU, A.Y. BRAGG-GRESHAM, J.L. VAN DONGEN, J. HUFFMAN, J.E. AHLUWALIA, T.S. CADBY, G. EKLUND, N. ERIKSSON, J. ESKO, T. FEITOSA, M.F. GOEL, A. GORSKI, M. HAYWARD, C. HEARD-COSTA, N.L. JACKSON, A.U. JOKINEN, E. KANONI, S. KRISTIANSSON, K. KUTALIK, Z. LAHTI, J. LUAN, J. MAGI, R. MAHAJAN, A. MANGINO, M. MEDINA-GOMEZ, C. MONDA, K.L. NOLTE, I.M. PERUSSE, L. PROKOPENKO, I. QI, L. ROSE, L.M. SALVI, E. SMITH, M.T. SNIEDER, H. STANCAKOVA, A. JU SUNG, Y. TACHMAZIDOU, I. TEUMER, A. THORLEIFSSON, G. VAN DER HARST, P. WALKER, R.W. WANG, S.R. WILD, S.H. WILLEMS, S.M. WONG, A. ZHANG, W. ALBRECHT, E. COUTO ALVES, A. BAKKER, S.J. BARLASSINA, C. BARTZ, T.M. BEILBY, J. BELLIS, C. BERGMAN, R.N. BERGMANN, S. BLANGERO, J. BLUHER, M. BOERWINKLE, E. BONNYCASTLE, L.L. BORNSTEIN, S.R. BRUINENBERG, M. CAMPBELL, H. CHEN, Y.I. CHIANG, C.W. CHINES, P.S. COLLINS, F.S. CUCCA, F. CUPPLES, L.A. D'AVILA, F. DE GEUS, E.J. DEDOUSSIS, G. DIMITRIOU, M. DORING, A. ERIKSSON, J.G. FARMAKI, A.E. FARRALL, M. FERREIRA, T. FISCHER, K. FOROUHI, N.G. FRIEDRICH, N. GJESING, A.P. GLORIOSO, N. GRAFF, M. GRALLERT, H. GRARUP, N. GRASSLER, J. GREWAL, J. HAMSTEN, A. HARDER, M.N. HARTMAN, C.A. HASSINEN, M. HASTIE, N. HATTERSLEY, A.T. HAVULINNA, A.S. HELIOVAARA, M. HILLEGE, H. HOFMAN, A. HOLMEN, O.et al. 2016. A principal component meta-analysis on multiple

anthropometric traits identifies novel loci for body shape. *Nature Communications,* 7**,** 13357.

RIPPE, V. DRIESCHNER, N. MEIBOOM, M. ESCOBAR, H.M. BONK, U. BELGE, G. & BULLERDIEK, J. 2003. Identification of a gene rearranged by 2p21 aberrations in thyroid adenomas. *Oncogene,* 22**,** 6111-6114.

RISCH, N. & MERIKANGAS, K. 1996. The future of genetic studies of complex human diseases. *Science,* 273**,** 1516-1517.

RITZ-TIMME, S. GABRIEL, P. TUTKUVIENE, J. POPPA, P. OBERTOVA, Z. GIBELLI, D. DE ANGELIS, D. RATNAYAKE, M. RIZGELIENE, R. BARKUS, A. & CATTANEO, C. 2011. Metric and morphological assessment of facial features: a study on three European populations. *Forensic Science International,* 207**,** 231-239.

RIVAL, J.M. & DAVID, A. 2001. The genetics of labio-palatal clefts. *Revue de Stomatologie et de Chirurgie Maxillo-Faciale,* 102**,** 171-181.

ROBERTS-HARRY, D. & SANDY, J. 2003. Orthodontics. Part 2: Patient assessment and examination I. *British Dental Journal,* 195**,** 489-493.

ROBERTSON, S.P. TWIGG, S.R. SUTHERLAND-SMITH, A.J. BIANCALANA, V. GORLIN, R.J. HORN, D. KENWRICK, S.J. KIM, C.A. MORAVA, E. NEWBURY-ECOB, R. ORSTAVIK, K.H. QUARRELL, O.W. SCHWARTZ, C.E. SHEARS, D.J. SURI, M. KENDRICK-JONES, J. & WILKIE, A.O. 2003. Localized mutations in the gene encoding the cytoskeletal protein filamin A cause diverse malformations in humans. *Nature Genetics,* 33**,** 487-491.

ROESSLER, E. DU, Y.Z. MULLOR, J.L. CASAS, E. ALLEN, W.P. GILLESSEN-KAESBACH, G. ROEDER, E.R. MING, J.E. RUIZ I ALTABA, A. & MUENKE, M. 2003. Loss-of-function mutations in the human GLI2 gene are associated with pituitary anomalies and holoprosencephaly-like features. *Proceedings of the National Academy of Sciences,* 100**,** 13424-13429.

ROGERS, C.R. MOONEY, M.P. SMITH, T.D. WEINBERG, S.M. WALLER, B.M. PARR, L.A. DOCHERTY, B.A. BONAR, C.J. REINHOLT, L.E. DELEYIANNIS, F.W.B. SIEGEL, M.I. MARAZITA, M.L. & BURROWS, A.M. 2009. Comparative Micro-Anatomy of the Orbicularis Oris Muscle between Chimpanzees and Humans: Evolutionary Divergence of Lip Function. *Journal of Anatomy,* 214**,** 36-44.

ROMITTI, P.A. SUN, L. HONEIN, M.A. REEFHUIS, J. CORREA, A. & RASMUSSEN, S.A. 2007. Maternal periconceptional alcohol consumption and risk of orofacial clefts. *American Journal of Epidemiology,* 166**,** 775-785.

ROSEN, H.M. 1991. Aesthetic refinements in genioplasty: the role of the labiomental fold. *Plastic and Reconstructive Surgery,* 88**,** 760-767.

SAHA, N. HIMANEN, J.P. & NIKOLOV, D.B. 2007. Cell-Cell Signaling via Eph Receptors and Ephrins. *Current Opinion in Cell Biology,* 19**,** 534-542.

SAMPSON, P.D. STREISSGUTH, A.P. BOOKSTEIN, F.L. LITTLE, R.E. CLARREN, S.K. DEHAENE, P. HANSON, J.W. & GRAHAM, J.M., JR. 1997. Incidence of fetal alcohol syndrome and prevalence of alcohol-related neurodevelopmental disorder. *Teratology,* 56**,** 317-326.

SANDLER, J. & MURRAY, A. 2002. Clinical photographs--the gold standard. *Journal of Orthodontics,* 29**,** 158-161.

SANDLER, P.J. MURRAY, A.M. & BEARN, D. 2002. Digital records in orthodontics. *Dental Update,* 29**,** 18-24.

SATOKATA, I. & MAAS, R. 1994. Msx1 deficient mice exhibit cleft palate and abnormalities of craniofacial and tooth development. *Nature Genetics,* 6**,** 348-356.

SAWYER, A.R. SEE, M. & NDUKA, C. 2009. 3D stereophotogrammetry quantitative lip analysis. *Aesthetic Plastic Surgery,* 33**,** 497-504.

SCARFE, W.C. & FARMAN, A.G. 2008. What is cone-beam CT and how does it work? *Dental Clinics of North America,* 52**,** 707-730.

SCHAAF, H. MALIK, C.Y. HOWALDT, H.P. & STRECKBEIN, P. 2009. Evolution of photography in maxillofacial surgery: from analog to 3D photography – an overview. *Clinical, Cosmetic and Investigational Dentistry,* 1**,** 39-45.

SCHUTTE, B.C. & MURRAY, J.C. 1999. The many faces and factors of orofacial clefts. *Human Molecular Genetics,* 8**,** 1853-1859.

SEAGER, D.C. KAU, C.H. ENGLISH, J.D. TAWFIK, W. BUSSA, H.I. & AHMED AEL, Y. 2009. Facial morphologies of an adult Egyptian population and an adult Houstonian white population compared using 3D imaging. *The Angle Orthodontist,* 79**,** 991-999.

SENDERS CW, P.E., HENDRICKX AG, CUKIERSKI MA 2003. Development of the upper lip. *Archives of Facial Plastic Surgery,* 5**,** 16-25.

SHIANG, R. LIDRAL, A.C. ARDINGER, H.H. BUETOW, K.H. ROMITTI, P.A. MUNGER, R.G. & MURRAY, J.C. 1993. Association of transforming growth-factor alpha gene polymorphisms with nonsyndromic cleft palate only (CPO). *The American Journal of Human Genetics,* 53**,** 836-843.

SI, J. YU, X. ZHANG, Y. & DEWILLE, J.W. 2010. Myc interacts with Max and Miz1 to repress C/EBPdelta promoter activity and gene expression. *Molecular Cancer,* 9**,** 92.

SITZMAN, T.J. COYNE, S.M. & BRITTO, M.T. 2015. The Burden of Care for Children With Unilateral Cleft Lip: A Systematic Review of Revision Surgery. *The Cleft Palate Craniofacial Journal* 53**,** 84-94.

SIVERTSEN, A. WILCOX, A.J. SKJAERVEN, R. VINDENES, H.A. ABYHOLM, F. HARVILLE, E. & LIE, R.T. 2008. Familial risk of oral clefts by morphological type and severity: population based cohort study of first degree relatives. *British Medical Journal,* 336**,** 432-434.

SLADEK, R. ROCHELEAU, G. RUNG, J. DINA, C. SHEN, L. SERRE, D. BOUTIN, P. VINCENT, D. BELISLE, A. HADJADJ, S. BALKAU, B. HEUDE, B. CHARPENTIER, G. HUDSON, T.J. MONTPETIT, A. PSHEZHETSKY, A.V. PRENTKI, M. POSNER, B.I. BALDING, D.J. MEYRE, D. POLYCHRONAKOS, C. & FROGUEL, P. 2007. A genome-wide association study identifies novel risk loci for type 2 diabetes. *Nature,* 445**,** 881-885.

SNEAD, M.P. YATES, J.R. WILLIAMS, R. PAYNE, S.J. POPE, F.M. & SCOTT, J.D. 1996. Stickler syndrome type 2 and linkage to the COL11A1 gene. *Annals of the New York Academy of Sciences,* 785**,** 331-332.

SOLOW, B. & TALLGREN, A. 1971. Natural Head Position in Standing Subjects. *Acta Odontologica Scandinavica,* 29**,** 591-607.

SONG, L. LI, Y. WANG, K. WANG, Y.-Z. MOLOTKOV, A. GAO, L. ZHAO, T. YAMAGAMI, T. WANG, Y. GAN, Q. PLEASURE, D.E. & ZHOU, C.J. 2009. Lrp6-mediated canonical Wnt signaling is required for lip formation and fusion. *Development,* 136**,** 3161-3171.

SOZEN, M.A. SUZUKI, K. TOLAROVA, M.M. BUSTOS, T. FERNANDEZ IGLESIAS, J.E. & SPRITZ, R.A. 2001. Mutation of PVRL1 is associated with sporadic, non-syndromic cleft lip/palate in northern Venezuela. *Nature Genetics,* 29**,** 141-142.

SPELIOTES, E.K. WILLER, C.J. BERNDT, S.I. MONDA, K.L. THORLEIFSSON, G. JACKSON, A.U. LANGO ALLEN, H. LINDGREN, C.M. LUAN, J. MAGI, R. RANDALL, J.C.

VEDANTAM, S. WINKLER, T.W. QI, L. WORKALEMAHU, T. HEID, I.M. STEINTHORSDOTTIR, V. STRINGHAM, H.M. WEEDON, M.N. WHEELER, E. WOOD, A.R. FERREIRA, T. WEYANT, R.J. SEGRE, A.V. ESTRADA, K. LIANG, L. NEMESH, J. PARK, J.H. GUSTAFSSON, S. KILPELAINEN, T.O. YANG, J. BOUATIA-NAJI, N. ESKO, T. FEITOSA, M.F. KUTALIK, Z. MANGINO, M. RAYCHAUDHURI, S. SCHERAG, A. SMITH, A.V. WELCH, R. ZHAO, J.H. ABEN, K.K. ABSHER, D.M. AMIN, N. DIXON, A.L. FISHER, E. GLAZER, N.L. GODDARD, M.E. HEARD-COSTA, N.L. HOESEL, V. HOTTENGA, J.J. JOHANSSON, A. JOHNSON, T. KETKAR, S. LAMINA, C. LI, S. MOFFATT, M.F. MYERS, R.H. NARISU, N. PERRY, J.R. PETERS, M.J. PREUSS, M. RIPATTI, S. RIVADENEIRA, F. SANDHOLT, C. SCOTT, L.J. TIMPSON, N.J. TYRER, J.P. VAN WINGERDEN, S. WATANABE, R.M. WHITE, C.C. WIKLUND, F. BARLASSINA, C. CHASMAN, D.I. COOPER, M.N. JANSSON, J.O. LAWRENCE, R.W. PELLIKKA, N. PROKOPENKO, I. SHI, J. THIERING, E. ALAVERE, H. ALIBRANDI, M.T. ALMGREN, P. ARNOLD, A.M. ASPELUND, T. ATWOOD, L.D. BALKAU, B. BALMFORTH, A.J. BENNETT, A.J. BEN-SHLOMO, Y. BERGMAN, R.N. BERGMANN, S. BIEBERMANN, H. BLAKEMORE, A.I. BOES, T. BONNYCASTLE, L.L. BORNSTEIN, S.R. BROWN, M.J. BUCHANAN, T.A.et al. 2010. Association analyses of 249,796 individuals reveal 18 new loci associated with body mass index. *Nature Genetics,* 42**,** 937-948.

STANIER, P. & MOORE, G.E. 2004. Genetics of cleft lip and palate: syndromic genes contribute to the incidence of non-syndromic clefts. *Human Molecular Genetics,* 13 Spec No 1**,** 73-81.

STANIER, P. & PAUWS, E. 2012. Development of the lip and palate: FGF signalling. *Frontiers of Oral Biology,* 16**,** 71-80.

SUN D, B.S., HAY ED. 2000. Epithelial-Mesenchymal Transformation Is the Mechanism for Fusion of the Craniofacial Primordia Involved in Morphogenesis of the Chicken Lip. *Developmental Biology,* 228 337-349.

SUTTIE, M. FOROUD, T. WETHERILL, L. JACOBSON, J.L. MOLTENO, C.D. MEINTJES, E.M. HOYME, H.E. KHAOLE, N. ROBINSON, L.K. RILEY, E.P. JACOBSON, S.W. & HAMMOND, P. 2013. Facial dysmorphism across the fetal alcohol spectrum. *Pediatrics,* 131**,** 779-788.

SUZUKI, K. & TSUCHIHASHI, Y. 1970. New attempt of personal identification by means of lip print. *Journal of the Indian Dental Association,* 42**,** 8-9.

SUZUKI, S. MARAZITA, M.L. COOPER, M.E. MIWA, N. HING, A. JUGESSUR, A. NATSUME, N. SHIMOZATO, K. OHBAYASHI, N. SUZUKI, Y. NIIMI, T. MINAMI, K. YAMAMOTO, M. ALTANNAMAR, T.J. ERKHEMBAATAR, T. FURUKAWA, H. DAACK-HIRSCH, S. L'HEUREUX, J. BRANDON, C.A. WEINBERG, S.M. NEISWANGER, K. DELEYIANNIS, F.W. DE SALAMANCA, J.E. VIEIRA, A.R. LIDRAL, A.C. MARTIN, J.F. & MURRAY, J.C. 2009. Mutations in BMP4 are associated with subepithelial, microform, and overt cleft lip. *The American Journal of Human Genetics,* 84**,** 406-411.

SUZUKI, T. LI, W. ZHANG, J.P. TIAN, Q.B. SAKAGAMI, H. USUDA, N. KONDO, H. FUJII, T. & ENDO, S. 2005. A novel scaffold protein, TANC, possibly a rat homolog of Drosophila rolling pebbles (rols), forms a multiprotein complex with various postsynaptic density proteins. *The European Journal of Neuroscience,* 21**,** 339-350.

SUZUKI, Y. JEZEWSKI, P.A. MACHIDA, J. WATANABE, Y. SHI, M. COOPER, M.E. VIET LE, T. NGUYEN, T.D. HAI, H. NATSUME, N. SHIMOZATO, K. MARAZITA, M.L. &

MURRAY, J.C. 2004. In a Vietnamese population, MSX1 variants contribute to cleft lip and palate. *Genetics in Medicine,* 6**,** 117-125.

T.C.S., C.G. 1996. Positional cloning of a gene involved in the pathogenesis of Treacher Collins syndrome. The Treacher Collins Syndrome Collaborative Group. *Nature Genetics,* 12**,** 130-136.

TANSATIT, T. APINUNTRUM, P. & PHETUDOM, T. 2014. A typical pattern of the labial arteries with implication for lip augmentation with injectable fillers. *Aesthetic Plastic Surgery,* 38**,** 1083-1089.

THEUSCH, E. BASU, A. & GITSCHIER, J. 2009. Genome-wide study of families with absolute pitch reveals linkage to 8q24.21 and locus heterogeneity. *The American Journal of Human Genetics,* 85**,** 112-119.

TOMA, A.M. ZHUROV, A. PLAYLE, R. ONG, E. & RICHMOND, S. 2009. Reproducibility of facial soft tissue landmarks on 3D laser-scanned facial images. *Orthodontics and Craniofacial Research,* 12**,** 33-42.

TOMA, A.M. ZHUROV, A.I. PLAYLE, R. MARSHALL, D. ROSIN, P.L. & RICHMOND, S. 2012. The assessment of facial variation in 4747 British school children. *The European Journal of Orthodontics,* 34**,** 655-664.

TOMPACH, P.C. WHEELER, J.J. & FRIDRICH, K.L. 1995. Orthodontic considerations in orthognathic surgery. *The International Journal of Adult Orthodontics and Orthognathic Surgery,* 10**,** 97-107.

TRASLER, D. 1968. Pathogenesis of cleft lip and its relation to embryonic face shape in A-J and C57BL mice. *Teratology,* 1**,** 33-49.

TROKOVIC, N. TROKOVIC, R. MAI, P. & PARTANEN, J. 2003. Fgfr1 regulates patterning of the pharyngeal region. *Genes & Development,* 17**,** 141-153.

TRUMPP, A. DEPEW, M.J. RUBENSTEIN, J.L. BISHOP, J.M. & MARTIN, G.R. 1999. Cre-mediated gene inactivation demonstrates that FGF8 is required for cell survival and patterning of the first branchial arch. *Genes & Development,* 13**,** 3136-3148.

TWIGG, S.R. KAN, R. BABBS, C. BOCHUKOVA, E.G. ROBERTSON, S.P. WALL, S.A. MORRISS-KAY, G.M. & WILKIE, A.O. 2004. Mutations of ephrin-B1 (EFNB1), a marker of tissue boundary formation, cause craniofrontonasal syndrome. *Proceedings of the National Academy of Sciences,* 101**,** 8652-8657.

USLU, V.V. PETRETICH, M. RUF, S. LANGENFELD, K. FONSECA, N.A. MARIONI, J.C. & SPITZ, F. 2014. Long-range enhancers regulating Myc expression are required for normal facial morphogenesis. *Nature Genetics,* 46**,** 753-758.

UTSUNO, H. KAGEYAMA, T. UCHIDA, K. YOSHINO, M. MIYAZAWA, H. & INOUE, K. 2010a. Facial soft tissue thickness in Japanese children. *Forensic Science International* 199**,** 109 e101-106.

UTSUNO, H. KAGEYAMA, T. UCHIDA, K. YOSHINO, M. OOHIGASHI, S. MIYAZAWA, H. & INOUE, K. 2010b. Pilot study of facial soft tissue thickness differences among three skeletal classes in Japanese females. *Forensic Science International,* 195**,** 165 e161-165.

VAN BAREN, M.J. VAN DER LINDE, H.C. BREEDVELD, G.J. BAARENDS, W.M. RIZZU, P. DE GRAAFF, E. OOSTRA, B.A. & HEUTINK, P. 2002. A double RING-H2 domain in RNF32, a gene expressed during sperm formation. *Biochemical and Biophysical Research Communications,* 292**,** 58-65.

VAN DEN ELZEN, M.E. TWIGG, S.R. GOOS, J.A. HOOGEBOOM, A.J. VAN DEN OUWELAND, A.M. WILKIE, A.O. & MATHIJSSEN, I.M. 2014. Phenotypes of

craniofrontonasal syndrome in patients with a pathogenic mutation in EFNB1. *European Journal of Human Genetics,* 22**,** 995-1001.

VEAU, V. Treatment of the unilateral harelip. Transcripts of 8th International Dental Congress Section XII, 1931 Paris. 126-130.

VEGA, H. WAISFISZ, Q. GORDILLO, M. SAKAI, N. YANAGIHARA, I. YAMADA, M. VAN GOSLIGA, D. KAYSERILI, H. XU, C. OZONO, K. JABS, E.W. INUI, K. & JOENJE, H. 2005. Roberts syndrome is caused by mutations in ESCO2, a human homolog of yeast ECO1 that is essential for the establishment of sister chromatid cohesion. *Nature Genetics,* 37**,** 468-470.

VENZA, I. VISALLI, M. PARRILLO, L. DE FELICE, M. TETI, D. & VENZA, M. 2011a. MSX1 and TGF-beta3 are novel target genes functionally regulated by FOXE1. *Human Molecular Genetics,* 20**,** 1016-1025.

VENZA, I. VISALLI, M. PARRILLO, L. DE FELICE, M. TETI, D. & VENZA, M. 2011b. MSX1 and TGF-β3 are novel target genes functionally regulated by FOXE1. *Human Molecular Genetics,* 20**,** 1016-1025.

VERLOES, A. DI DONATO, N. MASLIAH-PLANCHON, J. JONGMANS, M. ABDUL-RAMAN, O.A. ALBRECHT, B. ALLANSON, J. BRUNNER, H. BERTOLA, D. CHASSAING, N. DAVID, A. DEVRIENDT, K. EFTEKHARI, P. DROUIN-GARRAUD, V. FARAVELLI, F. FAIVRE, L. GIULIANO, F. GUION ALMEIDA, L. JUNCOS, J. KEMPERS, M. EKER, H.K. LACOMBE, D. LIN, A. MANCINI, G. MELIS, D. LOURENCO, C.M. SIU, V.M. MORIN, G. NEZARATI, M. NOWACZYK, M.J. RAMER, J.C. OSIMANI, S. PHILIP, N. PIERPONT, M.E. PROCACCIO, V. ROSELI, Z.S. ROSSI, M. RUSU, C. SZNAJER, Y. TEMPLIN, L. ULIANA, V. KLAUS, M. VAN BON, B. VAN RAVENSWAAIJ, C. WAINER, B. FRY, A.E. RUMP, A. HOISCHEN, A. DRUNAT, S. RIVIERE, J.B. DOBYNS, W.B. & PILZ, D.T. 2015. Baraitser-Winter cerebrofrontofacial syndrome: delineation of the spectrum in 42 cases. *European Journal of Human Genetics,* 23**,** 292-301.

VIEIRA, A.R. AVILA, J.R. DAACK-HIRSCH, S. DRAGAN, E. FELIX, T.M. RAHIMOV, F. HARRINGTON, J. SCHULTZ, R.R. WATANABE, Y. JOHNSON, M. FANG, J. O'BRIEN, S.E. ORIOLI, I.M. CASTILLA, E.E. FITZPATRICK, D.R. JIANG, R. MARAZITA, M.L. & MURRAY, J.C. 2005. Medical sequencing of candidate genes for nonsyndromic cleft lip and palate. *PLoS Genetics,* 1**,** 64.

VIG, P.S. & COHEN, A.M. 1979. Vertical growth of the lips: a serial cephalometric study. *American Journal of Orthodontics,* 75**,** 405-415.

VISSERS, L.E. VAN RAVENSWAAIJ, C.M. ADMIRAAL, R. HURST, J.A. DE VRIES, B.B. JANSSEN, I.M. VAN DER VLIET, W.A. HUYS, E.H. DE JONG, P.J. HAMEL, B.C. SCHOENMAKERS, E.F. BRUNNER, H.G. VELTMAN, J.A. & VAN KESSEL, A.G. 2004. Mutations in a new member of the chromodomain gene family cause CHARGE syndrome. *Nature Genetics,* 36**,** 955-957.

WAI-YEE LI, M.D., VESA KAARTINEN 2008. Signaling through Tgf-B type I receptor Alk5 is required for upper lip fusion. *Mechanisms of Development,* 125**,** 874-882.

WALLIS, D.E. & MUENKE, M. 1999. Molecular mechanisms of holoprosencephaly. *Molecular Genetics and Metabolism,* 68**,** 126-138.

WAN, M. & CAO, X. 2005. BMP signaling in skeletal development. *Biochemical and Biophysical Research Communications,* 328**,** 651-657.

WANG, H. CHEN, Y. HAN, J. MENG, Q. XI, Q. WU, G. & ZHANG, B. 2016. DCAF4L2 promotes colorectal cancer invasion and metastasis via mediating degradation of NFκb negative regulator PPM1B. *American Journal of Translational Research,* 8**,** 405-418.

WANG, H.Y. HSIEH, P.F. HUANG, D.F. CHIN, P.S. CHOU, C.H. TUNG, C.C. CHEN, S.Y. LEE, L.J. GAU, S.S. & HUANG, H.S. 2015. RBFOX3/NeuN is Required for Hippocampal Circuit Balance and Function. *Scientific Reports,* 5**,** 17383.

WARD, R.E. & JAMISON, P.L. 1991. Measurement precision and reliability in craniofacial anthropometry: implications and suggestions for clinical applications. *Journal of Craniofacial Genetics and Developmental Biology,* 11**,** 156-164.

WARMAN, M.L. CORMIER-DAIRE, V. HALL, C. KRAKOW, D. LACHMAN, R. LEMERRER, M. MORTIER, G. MUNDLOS, S. NISHIMURA, G. RIMOIN, D.L. ROBERTSON, S. SAVARIRAYAN, R. SILLENCE, D. SPRANGER, J. UNGER, S. ZABEL, B. & SUPERTI-FURGA, A. 2011. Nosology and Classification of Genetic Skeletal Disorders: 2010 Revision. *American Journal of Medical Genetics Part A,* 155**,** 943-968.

WATERHAM, H.R. & HENNEKAM, R.C. 2012. Mutational spectrum of Smith-Lemli-Opitz syndrome. *American Journal of Medical Genetics Part C,* 160C**,** 263-284.

WATERHAM, H.R. KOSTER, J. ROMEIJN, G.J. HENNEKAM, R.C. VREKEN, P. ANDERSSON, H.C. FITZPATRICK, D.R. KELLEY, R.I. & WANDERS, R.J. 2001. Mutations in the 3beta-hydroxysterol Delta24-reductase gene cause desmosterolosis, an autosomal recessive disorder of cholesterol biosynthesis. *The American Journal of Human Genetics,* 69**,** 685-694.

WEAVER, C.A. 2014. *Candidate gene analysis of 3D dental phenotypes in patients with malocclusion.* MS (Master of Science) Thesis, University of Iowa.

WEEDON, M.N. LANGO, H. LINDGREN, C.M. WALLACE, C. EVANS, D.M. MANGINO, M. FREATHY, R.M. PERRY, J.R. STEVENS, S. HALL, A.S. SAMANI, N.J. SHIELDS, B. PROKOPENKO, I. FARRALL, M. DOMINICZAK, A. JOHNSON, T. BERGMANN, S. BECKMANN, J.S. VOLLENWEIDER, P. WATERWORTH, D.M. MOOSER, V. PALMER, C.N. MORRIS, A.D. OUWEHAND, W.H. ZHAO, J.H. LI, S. LOOS, R.J. BARROSO, I. DELOUKAS, P. SANDHU, M.S. WHEELER, E. SORANZO, N. INOUYE, M. WAREHAM, N.J. CAULFIELD, M. MUNROE, P.B. HATTERSLEY, A.T. MCCARTHY, M.I. & FRAYLING, T.M. 2008. Genome-wide association analysis identifies 20 loci that influence adult height. *Nature Genetics,* 40**,** 575-583.

WEINBERG, S.M. BRANDON, C.A. MCHENRY, T.H. NEISWANGER, K. DELEYIANNIS, F.W. DE SALAMANCA, J.E. CASTILLA, E.E. CZEIZEL, A.E. VIEIRA, A.R. & MARAZITA, M.L. 2008a. Rethinking isolated cleft palate: evidence of occult lip defects in a subset of cases. *American Journal of Medical Genetics Part A,* 146A**,** 1670-1675.

WEINBERG, S.M. NAIDOO, S.D. BARDI, K.M. BRANDON, C.A. NEISWANGER, K. RESICK, J.M. MARTIN, R.A. & MARAZITA, M.L. 2009. Face shape of unaffected parents with cleft affected offspring: combining three-dimensional surface imaging and geometric morphometrics. *Orthodontics and Craniofacial Research,* 12**,** 271-281.

WEINBERG, S.M. NEISWANGER, K. RICHTSMEIER, J.T. MAHER, B.S. MOONEY, M.P. SIEGEL, M.I. & MARAZITA, M.L. 2008b. Three-dimensional morphometric analysis of craniofacial shape in the unaffected relatives of individuals with nonsyndromic orofacial clefts: a possible marker for genetic susceptibility. *American Journal of Medical Genetics Part A,* 146A**,** 409-420.

WERMKER, K. KLEINHEINZ, J. JUNG, S. & DIRKSEN, D. 2014. Soft tissue response and facial symmetry after orthognathic surgery. *Journal of Cranio-Maxillo-Facial Surgery,* 42**,** 339-345.

WIJAYAWEERA, C.J. AMARATUNGA, N.A. & ANGUNAWELA, P. 2000. Arrangement of the orbicularis oris muscle in different types of cleft lips. *The Journal of Craniofacial Surgery,* 11**,** 232-235.

WILKIE, A.O. SLANEY, S.F. OLDRIDGE, M. POOLE, M.D. ASHWORTH, G.J. HOCKLEY, A.D. HAYWARD, R.D. DAVID, D.J. PULLEYN, L.J. RUTLAND, P. & ET AL. 1995. Apert syndrome results from localized mutations of FGFR2 and is allelic with Crouzon syndrome. *Nature Genetics,* 9**,** 165-172.

WILKINSON, C.M. 2002. In vivo facial tissue depth measurements for white British children. *Journal of Forensic Sciences,* 47**,** 459-465.

WILKINSON, C.M. MOTWANI, M. & CHIANG, E. 2003. The relationship between the soft tissues and the skeletal detail of the mouth. *Journal of Forensic Sciences,* 48**,** 728-732.

WINDHAGER, S. SCHASCHL, H. SCHAEFER, K. MITTEROECKER, P. HUBER, S. WALLNER, B. & FIEDER, M. 2014. Variation at genes influencing facial morphology are not associated with developmental imprecision in human faces. *PLoS One,* 9**,** e99009.

WINKLER, C. KRUMSIEK, J. LEMPAINEN, J. ACHENBACH, P. GRALLERT, H. GIANNOPOULOU, E. BUNK, M. THEIS, F.J. BONIFACIO, E. & ZIEGLER, A.G. 2012. A strategy for combining minor genetic susceptibility genes to improve prediction of disease in type 1 diabetes. *Genes and Immunity,* 13**,** 549-555.

WONG, W.W. DAVIS, D.G. CAMP, M.C. & GUPTA, S.C. 2010. Contribution of lip proportions to facial aesthetics in different ethnicities: a three-dimensional analysis. *Journal of Plastic, Reconstructive & Aesthetic Surgery* 63**,** 2032-2039.

WOOD, A.R. ESKO, T. YANG, J. VEDANTAM, S. PERS, T.H. GUSTAFSSON, S. CHU, A.Y. ESTRADA, K. LUAN, J. KUTALIK, Z. AMIN, N. BUCHKOVICH, M.L. CROTEAU-CHONKA, D.C. DAY, F.R. DUAN, Y. FALL, T. FEHRMANN, R. FERREIRA, T. JACKSON, A.U. KARJALAINEN, J. LO, K.S. LOCKE, A.E. MAGI, R. MIHAILOV, E. PORCU, E. RANDALL, J.C. SCHERAG, A. VINKHUYZEN, A.A. WESTRA, H.J. WINKLER, T.W. WORKALEMAHU, T. ZHAO, J.H. ABSHER, D. ALBRECHT, E. ANDERSON, D. BARON, J. BEEKMAN, M. DEMIRKAN, A. EHRET, G.B. FEENSTRA, B. FEITOSA, M.F. FISCHER, K. FRASER, R.M. GOEL, A. GONG, J. JUSTICE, A.E. KANONI, S. KLEBER, M.E. KRISTIANSSON, K. LIM, U. LOTAY, V. LUI, J.C. MANGINO, M. MATEO LEACH, I. MEDINA-GOMEZ, C. NALLS, M.A. NYHOLT, D.R. PALMER, C.D. PASKO, D. PECHLIVANIS, S. PROKOPENKO, I. RIED, J.S. RIPKE, S. SHUNGIN, D. STANCAKOVA, A. STRAWBRIDGE, R.J. SUNG, Y.J. TANAKA, T. TEUMER, A. TROMPET, S. VAN DER LAAN, S.W. VAN SETTEN, J. VAN VLIET-OSTAPTCHOUK, J.V. WANG, Z. YENGO, L. ZHANG, W. AFZAL, U. ARNLOV, J. ARSCOTT, G.M. BANDINELLI, S. BARRETT, A. BELLIS, C. BENNETT, A.J. BERNE, C. BLUHER, M. BOLTON, J.L. BOTTCHER, Y. BOYD, H.A. BRUINENBERG, M. BUCKLEY, B.M. BUYSKE, S. CASPERSEN, I.H. CHINES, P.S. CLARKE, R. CLAUDI-BOEHM, S. COOPER, M. DAW, E.W. DE JONG, P.A. DEELEN, J. DELGADO, G.et al. 2014. Defining the role of common variation in the genomic and biological architecture of adult human height. *Nature Genetics,* 46**,** 1173-1186.

WRIGHT, M.J. & MARTIN, N.G. 2004. Brisbane Adolescent Twin Study: Outline of study methods and research projects. *Australian Journal of Psychology,* 56**,** 65-78.

YANG, Y. MCBRIDE, K.M. HENSLEY, S. LU, Y. CHEDIN, F. & BEDFORD, M.T. 2014. Arginine methylation facilitates the recruitment of TOP3B to chromatin to prevent R loop accumulation. *Molecular Cell,* 53**,** 484-497.

YI, J. SUN, Y. LI, Y. LI, C. LI, X. & ZHAO, Z. 2016. Cone-beam computed tomography versus periapical radiograph for diagnosing external root resorption: A systematic review and meta-analysis. *The Angle Orthodontist*.

YILDIRIM-TORUNER, C. SUBRAMANIAN, K. EL MANJRA, L. CHEN, E. GOLDSTEIN, S. & VITALE, E. 2004. A novel frameshift mutation of FOXC2 gene in a family with hereditary lymphedema-distichiasis syndrome associated with renal disease and diabetes mellitus. *American Journal of Medical Genetics Part A,* 131**,** 281-286.

YOON, Y.J. PERKIOMAKI, M.R. TALLENTS, R.H. BARILLAS, I. HERRERA-GUIDO, R. FONG, C.T. & KYRKANIDES, S. 2004. Transverse craniofacial features and their genetic predisposition in families with nonsyndromic unilateral cleft lip and palate. *The Cleft Palate Craniofacial Journal* 41**,** 256-261.

ZANKL, A. EBERLE, L. MOLINARI, L. & SCHINZEL, A. 2002. Growth charts for nose length, nasal protrusion, and philtrum length from birth to 97 years. *American Journal of Medical Genetics Part A,* 111**,** 388-391.

zANKL A, E.L., MOLINARI L, SCHINZEL A. 2002. . Growth charts for nose length, nasal protrusion, and philtrum length from birth to 97 years. *American Journal of Medical Genetics Part A,* 111**,** 388-391.

ZHAN, Q. CARRIER, F. & FORNACE, A.J., JR. 1993. Induction of cellular p53 activity by DNA-damaging agents and growth arrest. *Molecular and Cellular Biology,* 13**,** 4242-4250.

ZHANG, T.X. BEATY, T.H. & RUCZINSKI, I. 2012. Candidate pathway based analysis for cleft lip with or without cleft palate. *Statistical Applications in Genetics and Molecular Biology,* 11**,** 1-17.

ZHANG, Z. SONG, Y. ZHAO, X. ZHANG, X. FERMIN, C. & CHEN, Y. 2002. Rescue of cleft palate in Msx1-deficient mice by transgenic Bmp4 reveals a network of BMP and Shh signaling in the regulation of mammalian palatogenesis. *Development,* 129**,** 4135-4146.

ZHAO, F. SIU, M.K. JIANG, L. TAM, K.F. NGAN, H.Y. LE, X.F. WONG, O.G. WONG, E.S. CHAN, H.Y. & CHEUNG, A.N. 2011. Overexpression of dedicator of cytokinesis I (Dock180) in ovarian cancer correlated with aggressive phenotype and poor patient survival. *Histopathology,* 59**,** 1163-1172.

ZHOU, J. LU, Y. GAO, X.H. CHEN, Y.C. LU, J.J. BAI, Y.X. SHEN, Y. & WANG, B.K. 2005. The growth hormone receptor gene is associated with mandibular height in a Chinese population. *Journal of Dental Research,* 84**,** 1052-1056.

ZHU, L.Y. MENG, T. SHI, B. & DENG, D.Z. 2008. Anthropometric study of the upper lip of 1500 healthy children in Chengdu, Western China. *British Journal of Oral and Maxillofacial Surgery,* 46**,** 554-560.

ZHU, X.J. LIU, Y. YUAN, X. WANG, M. ZHAO, W. YANG, X. ZHANG, X. HSU, W. QIU, M. ZHANG, Z. & ZHANG, Z. 2016. Ectodermal Wnt controls nasal pit morphogenesis through modulation of the BMP/FGF/JNK signaling axis. *Developmental Dynamics,* 245**,** 414-426.